Department of Otorhinolaryngology
University of Helsinki
Finland

COCHLEAR GENE THERAPY;

VIRAL VECTORS, GENE TRANSFER, AND TREATMENT STRATEGIES FOR USHER SYNDROME

Laura Pietola

ACADEMIC DISSERTATION

To be presented, with permission of the Faculty of Medicine,
University of Helsinki, for public examination in
the Haartman Institute Lecture Hall 1, Haartmaninkatu 3,
on May 25th 2012, at 12 noon.

Helsinki 2012

SUPERVISED BY:

Docent Jussi Jero MD, PhD
Department of Otorhinolaryngology
University of Helsinki
Helsinki, Finland

Docent Antti A. Aarnisalo, MD, PhD
Department of Otorhinolaryngology
University of Helsinki
Helsinki, Finland

REVIEWED BY:

Docent Jorma Haapaniemi, MD, PhD
Department of Otorhinolaryngology
University of Turku
Turku, Finland

Docent Jing Zou, MD, PhD
Department of Otolaryngology
University of Tampere
Tampere, Finland

OFFICIAL OPPONENT:

Professor Anil K. Lalwani, MD, PhD
Vice-Chair for Research
Director of Division of Otology & Neurotology
Director of Columbia Cochlear Implant Center
Columbia University College of Physicians and Surgeons
New York, USA

ISBN 978-952-10-7969-6 (paperback)
ISBN 978-952-10-7970-2 (PDF)

Unigrafia Oy
Layout Tapio Kovero
Helsinki 2012

To my family

CONTENTS

LIST OF ORGINAL PUBLICATIONS

This thesis is based on the following original publications, which in the text are referred to by their Roman numerals.

I Pietola L, Aarnisalo AA, Abdel-Rahman AM, Västinsalo H, Isosomppi J, Löppönen H, Kentala E, Johansson R, Valtonen H, Vasama JP, Sankila EM, Jero J. Speech Recognition and Communication Outcomes with Cochlear Implantation in Usher Syndrome type III. Otol Neurotol. 2012 Jan;33(1):38-41.

II Aarnisalo AA, Aarnisalo P, Pietola L, Wahlfors J, Jero J. Efficacy of gene transfer through the round window membrane: an in vitro model. ORL J Otorhinolaryngol Relat Spec. 2006;68(4):220-7.

III Pietola L, Aarnisalo AA, Joensuu J, Pellinen R, Wahlfors J, Jero J. HOX-GFP and WOX-GFP lentivirus vectors for inner ear gene transfer. Acta Otolaryngol. 2008 Jun;128(6):613-20.

IV Pietola L, Jero J, Jalkanen R, Kinnari TJ, Jero O, Frilander M, Pajusola K, Salminen M, Aarnisalo AA. Effects of p27Kip1- and p53- shRNAs on kanamycin damaged mouse cochlea. World J of Otorhinolaryngol. 2012 Feb;2(1):1-7.

V Aarnisalo AA, Pietola L, Joensuu J, Isosomppi J, Aarnisalo P, Dinculescu A, Lewin AS, Flannery J, Hauswirth WW, Sankila EM, Jero J. Anti-clarin-1 AAV-delivered ribozyme induced apoptosis in the mouse cochlea. Hear Res. 2007 Aug;230(1-2):9-16.

ABBREVIATIONS

AAV	adeno-associated virus
Ad	adenovirus
bp	base pair
CAR	coxsackievirus and adenovirus receptor
CBA	chicken β-actin promoter
CI	cochlear implant
CKI	cyclin kinase inhibitor
CLRN1	clarin 1
CMV	cytomegalovirus promoter
COS	African green monkey kidney cells
CT	computerized tomography
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dB	decibel
E	embryonic day
EFS	human elongation factor 1-a promoter
FCS	fetal calf serum
GBI	Glasgow Benefit Inventory
GFP	green fluorescent protein
GHSI	Glasgow Health Status Inventory
GJB2	gap-junction protein connexin 26
GJB6	gap-junction protein, beta 6
HC	hair cell
HeLa	human cervical cancer cells
Hz	hertz
IHC	inner hair cell
ITR	inverted terminal repeat
LTR	long terminal repeat
MEF	mouse embryonic fibroblasts
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NSHL	non-syndromic hearing loss
OHC	outer hair cell
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde

pRb	retinoblastoma tumor suppressor protein
PTA	pure tone average
QOL	quality of life
RNA	ribonucleic acid
RP	retinitis pigmentosa
ROS	reactive oxygen species
RWM	round window membrane
Rz	ribozyme
SC	supporting cell
SGN	spiral ganglion neuron
shRNA	short hairpin ribonucleic acid
SIN	self-inactivating long terminal repeat
STAT1	signal transducer and activator of transcription-1
USH	Usher syndrome
USH1	Usher syndrome type 1
USH2	Usher syndrome type 2
USH3	Usher syndrome type 3
WPRE	Woodchuck hepatitis virus post-transcriptional element
wt	wild-type

ABSTRACT

Laura Pietola
"Cochlear gene therapy;
Viral vectors, gene transfer, and treatment strategies for Usher syndrome"
Department of Otorhinolaryngology, Faculty of Medicine,
University of Helsinki, Helsinki, Finland

Aims: The here presented studies focused on cochlear gene therapy and Usher syndrome type 3 (USH3). Study I evaluated the benefits of cochlear implantation in Finnish USH3 patients. Study II tested the feasibility of gene transfer through an intact round window membrane by means of an *in vitro* model. Study III concentrated on the evaluation of the efficiency and safety of lentivirus vectors in inner ear gene transfer. Study IV introduced three AAV-shRNAs (p27, p53, p27+53) and focused on exploring the effects of these constructs on the aminoglycoside-damaged mouse cochlea. Study V evaluated the effects of AAV-delivered anti-clarin-ribozyme on mouse cochlea.

Material and methods: Finnish USH3 patients answered three questionnaires which evaluated their quality of life after cochlear implantation. We also collected data of these patients' audiological tests and speech discrimination tests from their patient records. We developed an *in vitro* model suitable for cochlear gene transfer studies from a detached mouse round window membrane (RWM) and also tested the suitability of adeno-associated virus vectors and lentivirus vectors for cochlear gene therapy applications in cell lines and in the mouse cochlea.

Results: The audiological data collected from Finnish USH3 patients showed that most of our patients benefited from the cochlear implantation as much as implanted patients without visual deficits did. The Glasgow Benefit Inventory questionnaire had a positive score, which means that the patients believed that their health status had after the implantation improved.

The isolated mouse RWM was discovered to be a suitable model for the study of gene transfer *in vitro*. The permeability of the RWM was decreased by either damaging the cells with $AgNO_3$ and trichloracetic acid or by disrupting its cells' tight-junction expression with histamine-glycerol. However, there was no influence of these agents observed on the efficiency of the delivery of therapeutic agents or genes through the tested RWM.

GFP transgene delivered by lentivirus vectors was in the cochlea expressed in the structural lining cells of the perilymphatic space and in the epithelial cells surrounding the scala vestibuli and scala tympani. Structures of the organ of Corti showed no GFP expression. We evaluated the safety of lentivirus vectors

by inspecting peripheral organs for GFP expression, and cochlear sections for the presence of lymphocytes. GFP expression was found only in the liver of one mouse. Cochlear sections showed only few lymphocytes.

We studied the effects of AAV-delivered shRNAs on kanamycin-damaged mouse cochleae. AAV vector transduced inner and outer hair cells as well as supporting cells. Interestingly, we discovered that p53 and p27+53 shRNAs decreased the number of apoptotic cells in the cochleae. This may offer protection against kanamycin-induced cell death.

The AAV-GFP vector was expressed in the outer and inner hair cells, in some cells of the stria vascularis and in vestibular epithelial cells, but not in the spiral ganglion. After one month of expression a significantly increased number of apoptotic outer and inner hair cells and strial cells could be detected in the AAV-Rz group as compared to the AAV-GFP group. The results suggest that the anti-clarin-1 ribozyme may initiate a process which leads to apoptotic cell death in the cochlea. However, the detected apoptotic cell death in the AAV-Rz group could also be an unspecific effect due to an unspecific breakdown of mRNA, and not be related to clarin-1 loss.

Conclusions: Cochlear implantation is beneficial for USH3 patients and improves their quality of life. The detached mouse RWM model is suitable for inner ear gene transfer studies *in vitro*. Manipulation of the RWM with $AgNO_3$, trichloracetic acid or histamine-glycerol did not increase the permeability of the membrane. Lentivirus vectors are safe and can be used in gene transfer into the perilymph. Silencing of p53 protein may decrease apoptosis in the kanamycin-damaged mouse cochlea. AAV-delivered clarin-1 ribozyme may induce apoptosis in cochlear hair cells and cells of the stria vascularis. Apoptosis could explain the progressive nature of USH3.

INTRODUCTION

Hearing loss is the most common sensory deficit in humans. Genetic defects play a major part among patients with sensorineural hearing loss. It is estimated that one out of 1000 babies is born with a congenital hearing deficit and over half of the hearing loss cases detected in children in the prelingual state are caused by genetic factors. In adults, hearing loss can be caused by different factors, including noise or hazardous chemical exposure, but it is generally thought that also genetic factors have a role in adulthood hearing loss.

Usher syndrome (USH) is an autosomal recessive disorder defined by bilateral sensorineural hearing loss and a visual impairment phenotypically similar to retinitis pigmentosa (RP). USH is divided into three main clinical types (USH1, USH2 and USH3), based on the severity and progression of the hearing impairment, the presence or absence of vestibular dysfunction, and the age of onset of RP. The most common form of Usher syndrome in Finland is USH3, which comprises 40% of all USH cases, suggesting multiple founder effects. USH3 is caused by mutations in the clarin 1 (*CLRN1*) gene.

To date, treatment methods for sensorineural hearing loss are limited to rehabilitation with traditional hearing aids or cochlear implantation. An ideal cure would be targeted, long-term or permanent, and should cause as little damage as possible to the inner ear structure. Cochlear gene therapy studies have focused on the use of different types of viral and non-viral vectors. Gene transfer mediated by virus-derived vectors is efficient. Viral vectors can carry therapeutic genes and use the natural infectiousness of the virus in introducing and expressing genes within the target cells. Vector-based gene delivery has been carried out with adenoviruses, adeno-associated viruses (AAV), retroviruses, and herpes viruses. The safety of virus-based systems needs to be strictly evaluated before they can be considered for use in human applications. The risks involved in virus-based traetments are a possible distant spread outside the target and also immune reactions in the host.

The present study consists of five original publications. The studies focus on Usher syndrome III patients and different methods of cochlear gene therapy.

REVIEW OF THE LITERATURE

1 THE EAR AND HEARING

1.1 ANATOMY OF THE EAR

The ear is a peripheral auditory apparatus. It consists of three elements: outer ear, middle ear, and inner ear.

The outermost part of the outer ear is the pinna. It is composed of an elastic cartilage core covered by skin with hair follicles and sebaceous glands. The pinna collects sound waves and directs them to the auditory canal which is a passage extending from the auricle to the tympanic membrane (Figure 1A).

The middle ear, also called tympanic cavity, is an air-filled space in the temporal bone located between the tympanic membrane and the inner ear. The auditory ossicles, called malleus, incus, and stapes, are responsible for the sound transmission in the middle ear. They are arranged in a chainlike fashion and connected by small ligaments. The malleus is attached to the tympanic membrane and the footplate of the stapes is applied to the oval window, an opening of the bony labyrinth, at the other end of tympanic cavity. The auditory ossicles are kept joined by the tensor tympani and stapedius muscles. The tympanic membrane is oval-shaped with a conical depression near the center caused by the attachment of the malleus. The eustachian tube connects the middle ear with the nasopharynx (Figure 1A).

The inner ear is located within the temporal bone. It consists of a bony labyrinth. Inside the bony labyrinth is found the membranous labyrinth, a structure which includes both the vestibular and auditory systems. The vestibular system consists of two sacs called the utricle and the saccule, and three semicircular canals. The auditory system consists of the cochlear duct which is situated within a spiral bony canal anterior to the vestibular system. The membranous labyrinth contains endolymph, a fluid with a high concentration of K^+ (about 150 mM in humans) and low concentration of Na^+ (1 mM). Perilymph, a fluid with high Na^+ (140 mM) and low K^+ (7 mM), is present between the membranous labyrinth and the walls of the bony labyrinth (Kierszenbaum, 2002; Raphael and Altschuler, 2003) (Figure 1A).

The semicircular canals are located within the bony labyrinth. The three ducts (horizontal, superior and posterior) are connected to the utricle. The endolymphatic duct is formed from joined ducts originating from the utricle and the saccule. The

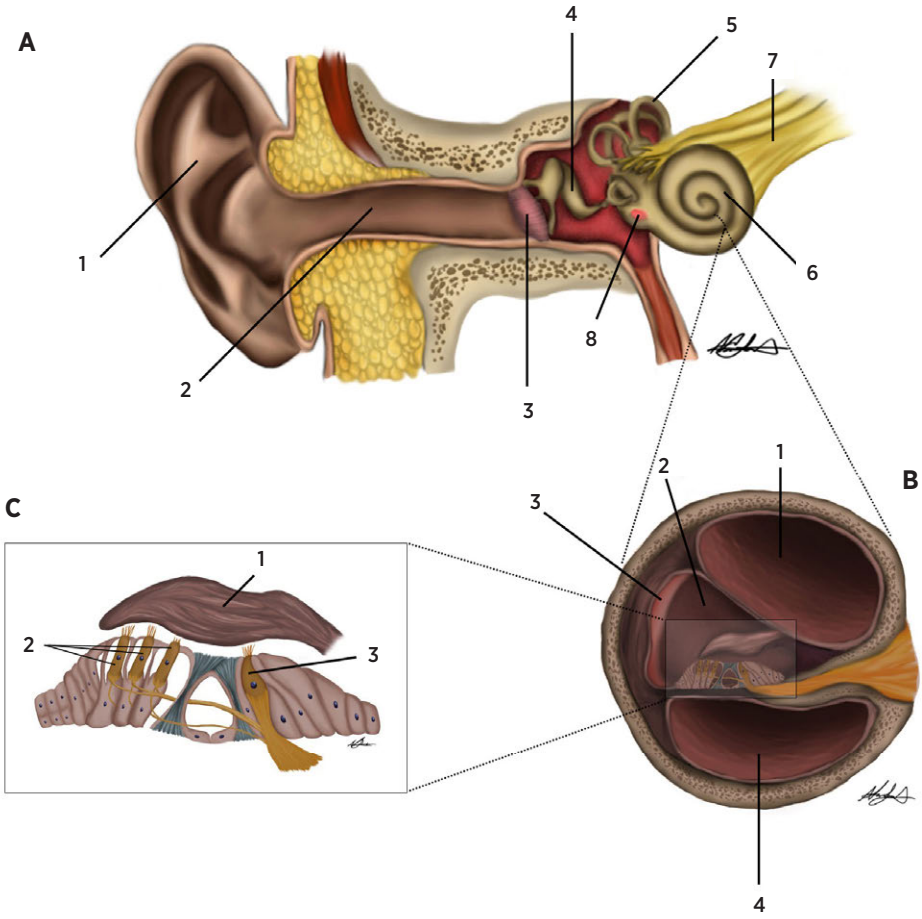


Figure 1. The ear

A) Anatomy of the ear

1. pinna, 2. auditory canal, 3. tympanic membrane, 4. ossicles,
5. semicircular canals, 6. cochlea, 7. 8th cranial nerve, 8. round window

B) The cochlea

1. scala vestibuli, 2. scala media, 3. stria vascularis, 4. scala tympani

C) The organ of Corti

1. tectorial membrane, 2. outer hair cells, 3. inner hair cell

endolymphatic duct ends in the endolymphatic sac, located between the layers of the meninges. Small dilations, called ampullae, are found close to the sites where the semicircular ducts are connected to the utricle. Each ampulla contains a ridge called the crista ampullaris. The sensory epithelium is covered by a gelatinous mass called the cupula and located within the crista ampullaris (Kierszenbaum, 2002). The sensory epithelium in the semicircular canals consists of two cell types, the hair cells and the supporting cells.

1.2 THE COCHLEA

The cochlea has three parallel spiraling chambers. The cochlear duct, also called the scala media, is the central chamber and contains endolymph. The cochlear duct is a membranous coiled duct inside the bony cochlea. It has an apex and a base, and the duct spirals for about two and two-thirds turns. In a cross section, there is along the bottom of the cochlear duct the basilar membrane, above the cochlear duct is Reissner's membrane, and laterally there is the stria vascularis. Endolymph is produced by the cells and capillaries of the stria vascularis. The spiral-shaped bony core of the cochlea is the modiolus. Above the cochlear duct and along it runs the scala vestibuli, starting at the oval window. Below the cochlear duct run the scala tympani, ending at the round window. The scala vestibuli and the scala tympani meet at an opening, called the helicotrema, at the apex of the cochlea (Figure 1B).

The sensory epithelium of the cochlea is situated in the organ of Corti. This is formed by the inner and the outer hair cells, supporting cells, the tectorial membrane and the inner tunnel (Raphael and Altschuler, 2003). The inner tunnel is bordered by the outer and the inner pillar cells, which together separate the inner hair cells from the outer hair cells (Kierszenbaum, 2002). The inner hair cells are completely surrounded by supporting cells but the outer hair cells' bodies stand free and are surrounded by endolymph except at their apical and basal poles (Lim and Kalinec, 1998).

A single line of inner hair cells extends along the cochlea from the base to the apex. The outer hair cells are arranged in three parallel rows and they also extend from the base to the apex. A hair bundle, formed by stereocilia, extends from the apical part of each hair cell (Hudspeth, 1989). The stereocilia contain many fine longitudinal actin filaments. The stereocilia of a hair cell are arranged in order of increasing length from one side to the other (Raphael and Altschuler, 2003) (Figure 1C).

1.3 THE ROUND WINDOW MEMBRANE (RWM)

The round window is an opening onto the inner ear and it is closed off from the middle ear by the round window membrane (RWM). The RWM consist of three layers: an outer epithelium, a core of connective tissue, and an inner epithelium. The outer epithelium faces the middle ear and consists of a single layer of cuboidal cells. Tight junctions can be observed near the surface of these epithelial cells. The connective tissue core layer consists of fibroblasts, collagen, elastic fibers, and blood and lymph vessels. The inner epithelium faces the inner ear and is composed of squamous cells with long lateral extensions (Goycoolea and Lundman, 1997). The average thickness of the human RWM is 70µm, whereas the average thickness of the RWM of rodents is 10-14µm (Goycoolea et al. 1988).

Early studies on the RWM suggested that its role was to release mechanical energy and/or conduct sound to the scala tympani (Wever and Lawrens, 1948). After learning more aboout the anatomy of the RWM, it has been postulated that the RWM could also be involved in secretion and/or absorption (Miriszlai and Benedeczky, 1978; Richardson et al., 1971). Especially the outer epithelium, with microvilli and abundant cell organelles such as mitochondria, rough endoplasmic reticulum and Golgi complex, should be able to play a part in metabolic activities and transport.

The permeability of the RWM has been studied extensively in animal experiments. These studies have shown that even though the RWM is three-layered it behaves more like a semipermeable membrane. Antibiotics, antiseptics, arachidonic acid metabolites, local anesthetics, toxins, albumin, cationic ferritin, horseradish-peroxidase, 1µm latex spheres, and neomycin-gold spheres have been shown to pass through it. The permeability of the RWM is influenced by factors such as size, configuration, concentration, liposolubility and electric charge of the passing material, and the thickness of the membrane (Goycoolea et al., 1988). Transfer through the RWM can occur by diffusion through the cytoplasm (e.g. exotoxin), in pinocytotic vesicles (e.g. cationic ferritin), or through channels between cells (e.g. latex spheres) (Goycoolea, 1995).

Inflammation in the middle ear mucosa causes changes in the permeability of the RWM. It decreases during inflammation due to swelling in the RWM (Goycoolea, 1992). These changes protect the inner ear during inflammation. The permeability of the RWM seems to decrease after $AgNO_3$, trichloracetic acid and histamine-glycerol treatments, thus, these will not enhance the delivery of therapeutic agents or transgenes into the inner ear through an otherwise intact RWM (Aarnisalo et al., 2006). A recent study by Wang et al. showed that administration of collagenase I or II to the RWM of a guinea pig partially digested the membrane and allowed adeno-associated virus (AAV) vectors to pass though the RWM *in vivo* (Wang et al. 2011).

1.4 SOUND TRANSDUCTION

Sounds are pressure changes that spread through the air. A sound's frequency is measured in Hertz (Hz) and its intensity in decibels (dB). Sound is a mixture of pure tones and each pure tone results from sinusoidal waves of a particular frequency. Pure tones are characterized by their frequency, but also by their amplitude and phase. Normal human ear is sensitive to pure tones with frequencies between 20 to 20000 Hz. The main frequencies used during speech are between 300 and 3500 Hz with an intensity of 65 dB (Berne and Levy, 2003). Sounds exceeding 85 dB may cause damage to the auditory system. The ear detects sound waves traveling in air but the neural transduction mechanism detects fluid movements within the cochlea (Berne and Levy, 2003). The acoustic impedance of water is much higher than that of air which is the reason for why the ear needs a special apparatus for impedance matching (Berne and Levy, 2003).

Sound waves pass through the auditory canal to the tympanic membrane. The moving sound pressure wave causes an inward movement of the membrane. This causes the chain of ossicles (malleus, incus, and stapes) to move. The footplate of the stapes pushes into the membrane of the oval window, the movement of which moves the perilymph within the scala vestibuli. The pressure wave moves within the perilymph and is transmitted through the basilar membrane of the cochlea to the scala tympani. As a consequence of this the membrane of the round window bulges into the middle ear. The tympanic membrane and the chain of ossicles function as an impedance matching device (LeMasurier and Gillespie, 2005). Factors which influence this impedance matching are: 1) the ratio of the surface area of the tympanic membrane to that of the oval window, and 2) the mechanical advantages of the lever system formed by the ossicle chain (Berne and Levy, 2003). The movement of the fluid in the cochlea leads to vibrations of the basilar membrane which causes the hair cells of the organ of Corti to move with respect to the overlying tectorial membrane. This movement of the hair cells causes their stereocilia to be bent, which in turn opens ion channels. As a result the hair cells excite the primary afferent neurons whose axons run in the 8_{th} cranial nerve (Lim and Kalinec, 1998; Raphael and Altschuler, 2003).

A hair cell is a mechanoreceptor. This receptor uses the energy contained in a mechanical stimulus to open the ion channels which produce an electrical response (Hudspeth, 1989). The basilar membrane's vibration stimulates hair cells and this initiates mechanotransduction. Traveling waves of different frequencies peak at different positions along the basilar membrane, as a result each excite a certain subset of hair cells. The actual transformation of the mechanical stimuli to electrical signals occurs in hair bundles. The bundle is extremely sensitive to mechanical stimuli; variations of less than the diameter of an atom are sufficient to initiate mechanotransduction (LeMasurier and Gillespie, 2005). The key to the hair bundles' function is their form (Hudspeth, 2005). At the tip of a stereocilium, two or three

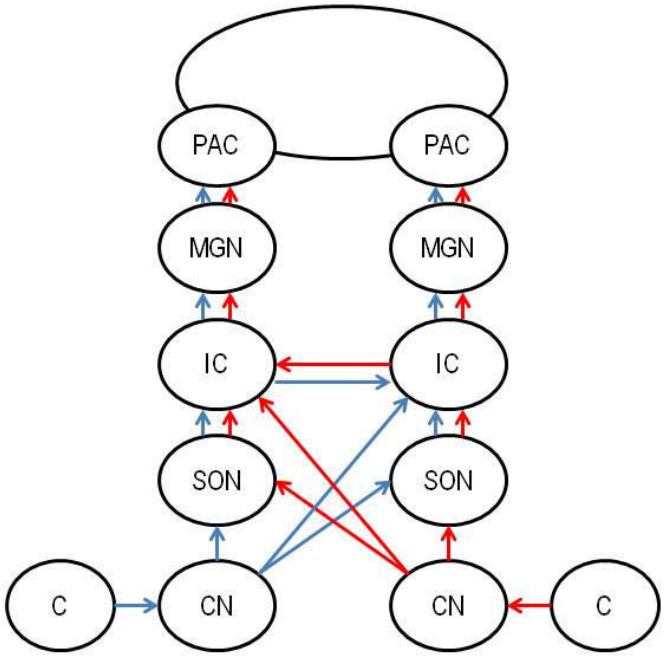


Figure 2. Schematic presentation of the auditory pathways

C: cochlea; CN: cochlear nuclei; SON: superior olivary nuclei; IC: inferior colliculus; MGN: medial geniculate nucleus; PAC: primary auditory cortex

molecular filaments extend diagonally upward to an insertion on the side of the longest adjacent stereocilium. The stereociliary tips are pulled together by tension on the elastic tip links. When the hair bundle moves toward its tall edge, the motion between adjacent stereocilia increases the tension in each tip link. This is defined as a positive stimulus and the tension in the tip link opens one or a few ion channels, allowing an influx of K^+ and Ca^{2+} and causing a cellular depolarization (Hudspeth, 2005). The influx of K^+ is a manifestation of the unusually high K^+ concentration in the endolymph. The receptor current is furthermore enhanced by the endolymph's extracellular potential of +80 mV, which gives rise to a +150 mV electrical driving force for K^+ and Ca^{2+} entry (LeMasurier and Gillespie, 2005). As hair cells depolarize, voltage-dependent Ca^{2+} channels near the basolateral synapses open. Increased Ca^{2+} levels stimulate the neurotransmitter release at the glutamatergic synapses, initiating signal transmission to afferent neurons (LeMasurier and Gillespie, 2005; Ottersen et al., 1998).

1.5 AUDITORY CIRCUITS

Hair cells (HCs) transmit the information about the timing, frequency and intensity of sounds to ribbon synapses of the spiral ganglion neurons. The primary receptor cells are the inner hair cells (IHCs) which lie closest to the spiral ganglion cell bodies. The outer hair cells' (OHCs') role is to improve the sensitivity of sound detection. Spiral ganglion neurons (SGN) are subdivided into two classes: Type I and Type II. In the mouse, each IHC connects with 10–20 Type I SGNs, but each Type I SGN receives input from only one hair cell (Meyer et al., 2009). The Type I SGNs' axons project into the hindbrain, where they bifurcate and make connections with multiple cells in the cochlear nucleus (Fekete et al., 1984; Ryugo, 2008). Type II SGNs connect to multiple OHCs. In the central nervous system, the Type II SGNs axons terminate within the small cell cap in the cochlear nucleus (Brown et al., 1988). Even though it is known that Type II SNGs receive synaptic input from OHCs, their actual function is poorly understood (Weisz et al., 2009).

In the cochlea, HCs and neurons are topographically arranged according to sound frequency: HCs located at the basal part of the cochlea detect higher frequencies while HCs located at the apical part detect lower ones. This tonotopic structure of the cochlea is preserved in the arrangement of the SGNs, in the organization of the central projections into the hindbrain, and throughout every stage of auditory processing from brainstem to the cortex (Kandler et al., 2009).

From the cochlear nuclei the input goes to the superior olivary nuclei, where the first binaural interactions occur, and to the inferior colliculus. Axons from cells of the inferior colliculus go to the medial geniculate nucleus in the thalamus. The axons from the geniculate nucleus terminate in the primary auditory cortex (Kandell, Schwartz, Jessell, 2000). (Figure 2)

1.6 DEVELOPMENT AND STRUCTURE OF THE MOUSE COCHLEA

The mouse inner ear develops from a thickening of the ectoderm known as the otic placode. On embryonic day (E) 10, a tube-like structure known as the endolymphatic duct projects dorsally from the medial part of the otocyst. On E12, the cochlea adopts a more elaborate shape consisting of a proximal and a distal part. The proximal part of the cochlea expands further ventromedially and the distal part continues to coil. The development of the cochlea ends on E15 (Morsli et al., 1998).

The mouse cochlea is structurally similar to the human cochlea. The evident difference is its size. For example, the volume of human endolymph is 7.7 µl and of the perilymph 79.5 µl whereas the volume of mouse endolymph is only 0.19 µl and of the perilymph 0.62 µl (Jahn and Santos-Sacchi, 2001). Since the molecular basis of hearing is poorly understood, it is at the moment difficult to investigate the differencies in hearing at the molecular level between humans and mice any further.

2 HEREDITARY HEARING LOSS

Hearing loss is the most common birth defect and affects an estimated 22.5 million Europeans (Dror and Avraham 2009). Approximately 8000 Finnish persons are deaf (The Finnish Association of the Deaf; www.kl-deaf.fi). One of every 500 newborns shows bilateral permanent sensorineural hearing loss, and by adolescence the prevalence increases to 3.5. per 1000 (Morton and Nance 2006). Over half of the prelingual deafness cases are genetic, and these most often autosomal recessive and nonsyndromic. Half of the nonsyndromic autosomal recessive hearing loss cases can be attributed to the disorder DFNB1, which is caused by mutations in the *GJB2* gene (DFNB1A) and the *GJB6* gene (DFNB1B). A small percentage of the prelingual deafness cases are syndromic or autosomal dominant nonsyndromic. In the general population the prevalence of hearing loss increases with age. In the elderly, hearing loss can be caused by different factors, for example environmental ones, but genetics have some influence on it.

Hearing loss can be classified in different ways. Typically the classification is made according to the cause of the hearing loss or by its normal characteristics such as age of onset or severity (Gürtler and Lalwani, 2002). The most common way to classify hereditary hearing losses is to determine whether the hearing loss is syndromic or nonsyndromic (Gürtler and Lalwani, 2002). Patients with syndromic hearing loss have an inherited hearing impairment together with some other clinical abnormalities, whereas patients with nonsyndromic hearing loss show only impaired hearing. Both phenotypes, syndromic and nonsyndromic, can result from mutations in the same gene (Keats and Berlin, 1999). This represents the heterogeneity of hereditary hearing loss.

In order to function properly, the ear needs to have its normal anatomical structure. However, there are many genes involved in hearing, and the genetics of hearing loss is extraordinarily complex (Schrijver and Gardner, 2006). A rough estimate is that in the biology of hearing there are several hundred genes involved (Friedman and Griffith, 2003). Inherited hearing loss can be autosomal recessive or dominant, X-linked, or mitochondrial. Mitochondrial mutations are inherited from the mother and the mutation is present in almost all mitochondria (Schrijver and Gardner, 2006). It has been thought that mitochondrial mutations are also involved in the progressive hearing loss associated with aging (Sinnathuray et al., 2003) as well as in both syndromic and nonsyndromic forms of hearing loss.

2.1 NONSYNDROMIC AND SYNDROMIC HEARING LOSS

Most of the hereditary hearing loss cases (~70%) are nonsyndromic. The pattern of inheritance in hereditary hearing loss can be autosomal recessive, autosomal

dominant, X-liked, Y-linked, or mitochondrial. Autosomal recessive inheritance accounts for 80% of the nonsyndromic hearing loss (NSHL) cases and is the most common cause of genetic deafness (Petersen and Willems, 2006). Autosomal dominant inheritance is less common, accounting for 20% of the cases (Petersen, 2002). Sex-linked (Petersen et al., 2008) and mitochondrial (Kokotas et al., 2007) forms are much rarer. X-linked inheritance accounts for 1-5% of genetic hearing loss cases. Y-linked inheritance has been described by Wang in an extended seven generation Chinese pedigree (Wang, 2004). It has been estimated that mitochondrial inheritance is responsible for less than 1% of hearing loss cases (Kokotas et al., 2007). The phenotypes of different forms of inherited hearing loss vary strongly. People displaying nonsyndromic autosomal-recessive inheritance show prelingual onset and the hearing loss is from severe to profound due to cochlear defects (Petersen and Willems, 2006). Nonsyndromic autosomal-dominant forms show a less severe phenotype. The onset is typically postlingual, the severity is from moderate to severe, and the hearing loss is progressive (Petersen, 2002). In X-linked traits the onset is in males earlier and the hearing loss also more severe than in females who are carriers of the trait (Petersen et al., 2008). The Y-linked form affects only males, the onset is postlingual, and the hearing loss is from mild to severe and progressive (Petersen et al., 2008). In mitochondrial hearing loss the onset is postlingual, the severity ranges from normal hearing to profound deafness, and the hearing loss is usually progressive (Kokotas et al., 2007).

Individuals with NSHL show all the same phenotype, which makes it hard to identify all the different genes responsible. Because of the uniform phenotype it was thought for a long time that nonsyndromic deafness resulted from genes which were only expressed in the cochlea (Gürtler and Lalwani, 2002). It is possible to divide deafness-associated genes into groups on the basis of the proteins they encode and the functions they have. These groups include extracellular matrix proteins, cytoskeletal components, transcription factors, cellular trafficking proteins, proteins involved in ion homeostasis, receptors, and proteins of unknown function (Gürtler and Lalwani, 2002; Schrijver and Gardner, 2006).

The first recessive nonsyndromic deafness locus DFNB1 was found in 1994 in chromosome 3q11-q12 (Guilford et al., 1994). DFNB1A is caused by a mutation in the *GJP2* gene. The *GJP2* gene encodes connexin 26, a gap-junction protein of the β-group which has a role in ion transportation between cells in the cochlea (Schrijver and Gardner, 2006). Mutations in the *GJP2* gene are responsible for over 50% of the autosomal-recessive deafness cases in the USA and Europe (Schrijver and Gardner, 2006). Today, at least of 64 genes have been identified and 125 loci been mapped for autosomal recessive and autosomal dominant hearing loss; three genes and 5 loci are associated with X-linked inheritance; the gene associated with Y-linked inheritance is still unknown, but one locus has been mapped; 11 genes are

associated with mitochondrial inheritance (Hereditary Hearing Loss Homepage; http://hereditaryhearingloss.org).

15-30% of all hereditary hearing loss cases are syndromic. There are hundreds of reported syndromes with impaired hearing as a symptom (Dror and Avraham, 2009). The inheritance patterns of syndromic hereditary hearing loss can be classified into three distinguishable groups. The first group is comprised of syndromes with cytogenetic or chromosomal anomalies, the second group is characterized by syndromes which are transmitted by classical monogenic or Mendelian inheritance, and the third group shows syndromes with multifactorial influences in which the phenotype of the syndrome results from a mixture of genetic and environmental factors (Gorlin et al., 1995). Usher Syndrome (Saihan et al., 2009), Goldenhar Syndrome, Treacher Collins Syndrome (Horbelt CV, 2008), CHARGE (Sanlaville and Verloes, 2007), Pendred Syndrome (Glaser B, 2003), Jervell and Lange-Nielsen Syndrome (Bitner-Glindzicz and Tranebjaerg, 2000), Stickler Syndrome (Admiraal et al.,2002), Waardenburg Syndrome (Read AP, 2000), Branchio-Oto-Renal Syndrome (Kochhar et al., 2007), Norrie disease (Berger W, 1998), and Alport Syndrome (Kashtan CE 1999) are all examples of syndromes associated with hearing impairment.

2.2 USHER SYNDROME

Usher syndrome (USH) is an autosomal recessive disorder which is defined by bilateral sensorineural deafness and retinitis pigmentosa, a progressive degeneration of the retina that leads to loss of night vision, restriction of the visual field and blindness (Bayazit and Yilmar, 2006; Keats and Savas, 2004; Petit C., 2001). In addition, variable vestibular dysfunctions are also related to it (Pakarinen, 1995). The prevalence of Usher syndrome is 3.5- 6.2 per 100 000 in different populations worldwide (Saihan et al., 2009, Yan and Liu, 2010). Over half of the individuals who are both deaf and blind are afflicted with Usher syndrome (Boughman et al., 1983).

2.2.1 Usher syndrome subtypes

Usher syndrome is clinically heterogeneous and it has been divided into three subtypes: Usher type I (USH1), type II (USH2) and type III (USH3).

USH1 is the most severe form and characterized by severe to profound congenital sensorineural deafness, constant dysfunction of the vestibular system and onset of retinitis pigmentosa in childhood (Petit C, 2001).

Individuals with USH2 show a mild hearing loss for low frequency and severe hearing loss for high frequency sounds; vestibular dysfunction is absent. The visual

impairment develops progressively and the onset of retinitis pigmentosa occurs in puberty. Over half of all USH patients have USH2 (Eudy et al., 1998; Petit C, 2001).

In USH3 the hearing loss is progressive and the individuals may suffer from vestibular dysfunction. The onset of retinitis pigmentosa and the degree of vestibular dysfunction vary among those affected (Keats and Savas, 2004). USH3 is rarest from of the syndrome in most populations except in Finland (Pakarinen et al., 1995) and among Ashkenazi Jews in various regions (Ness et al., 2003). In Finland USH3 is the most common form of Usher syndrome (Figure 3). It comprises 40% of all Finnish USH cases, suggesting multiple founder effects (Karjalainen et al., 1989; Pakarinen et al., 1995).

2.2.2 USH genes

To this day, 12 chromosomal loci with nine different USH gene products have been identified:

- USH1B is caused by mutation in *MYO7* (encodes protein myosin VIIa) (Weil et al., 1995 nature)
- USH1C by mutation in *USH1C* (encodes harmonin) (Verpy et al., 2000)
- USH1D by mutation in *CDH23* (encodes cadherin 23) (Bolz H et al., 2001)
- USH1E by unknown gene (Chaïb 1997)
- USH1F by mutation in PCDH15 (encodes protocadherin 15) (Ahmed et al., 2001; Alagramam et al., 2001; Wayne et al., 1996)
- USH1G by mutation in *USH1G* (encodes scaffold protein containing ankyrin repeats and SAM domain [SANS]) (Weil et al., 2003)
- USH1H by unknown gene (Ahmed et al. 2009)
- USH2A by mutation in *USH2A* (encodes usherin) (Eudy et al., 1998; van Wijk et al., 2004)
- USH2C by mutation in *VLGR1* (encodes very large G-protein-coupled receptor 1) (Weston et al., 2004);
- USH2D by mutation in *WHRN* (encodes whirlin) (Ebermann et al., 2007)
- USH3A by mutation in *CLRN1* (encodes clarin 1) (Adato et al., 2002; Joensuu et al., 2001; Sankila et al., 1995).
- USH3B by unknown gene (Chaïb 1997)

The *USH1A* locus was reported in 1992 (Kaplan et al., 1992) but was demonstrated to be a linkage artifact in 2006 (Gerber et al., 2006). *PDZD7* is a USH modifier gene. Mutated *PDZD7* affects the USH phenotype only when combined with mutated *USH2A* or *VLGR1b* (Ebermann et al., 2010).

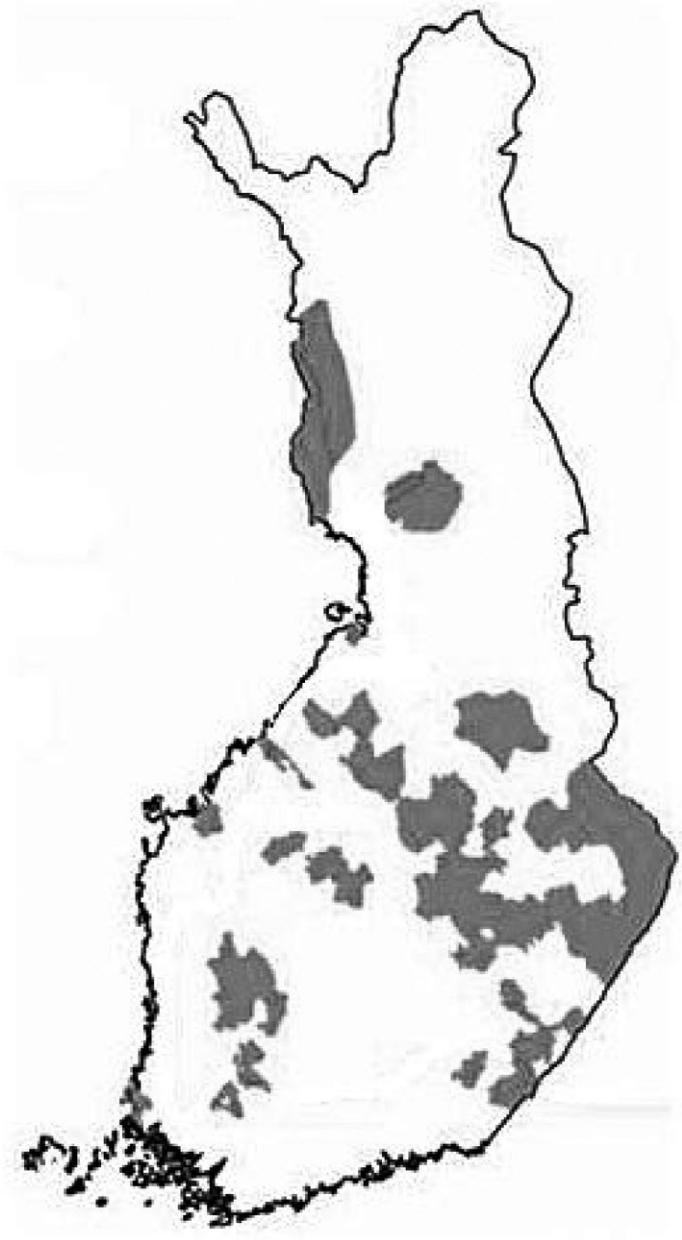


Figure 3. Usher syndrome type III

Map of Finland showing the birthplaces of USH3 patients' grandparents

It has been suggested that USH proteins form a network which has a critical role in the development and maintenance of the sensorineural cells in both the inner ear and the retina (Adato et al., 2005; Kremer et al., 2006; Maerker et al., 2008; Reiners et al., 2005, 2006; Tian et al., 2009). The functions of the USH proteins are, though, still poorly known.

2.2.3 USH3

USH3 is caused by mutations in the *CLRN1* gene, which encodes transmembrane protein clarin 1 (CLRN1). CLRN1 is assumed to have four transmembrane domains, and it shares a homology with tetraspanin family (Adato et al., 2002) (Figure 4). It is predicted to be related with ribbon synapses of cochlear hair cells and photoreceptors, and has been suspected to be involved with the cytoskeleton (Geng et al., 2009; Tian et al., 2009; Zallocchi et al. 2009, 2012). Eighteen *CLRN1* mutations have been documented (Aller et al., 2004; Eberman et al., 2007; Herrera et al., 2008; Joensuu et al., 2001; Ness et al., 2003; Sadeghi et al., 2005).

Finnish USH3 patients show two mutations: p.Y176X (Finnish founder mutation) and p.M120K (in combination with the founder mutation). In USH3 patients in Ashkenazi Jews the founder mutation is p.N48K (Adato et al., 2002; Fields et al., 2002; Ness et al., 2003).

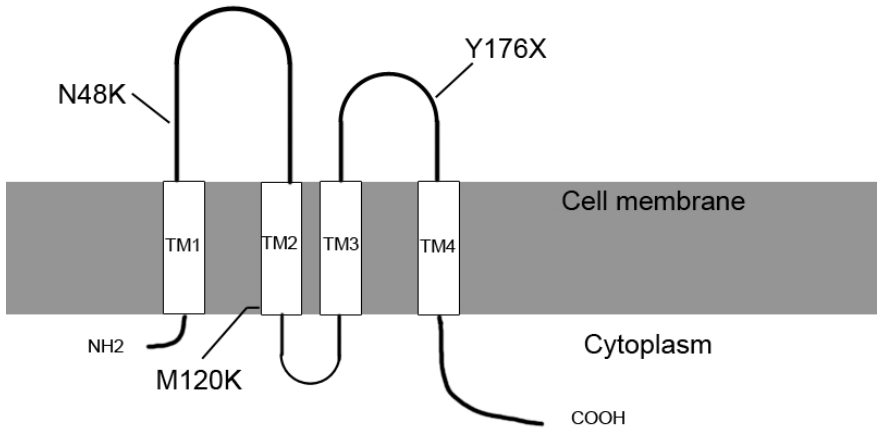


Figure 4. Schematic presentation of clarin 1

TM: transmembrane domain

2.3 CURRENT TREATMENT OPTIONS FOR HEREDITARY HEARING LOSS

Currently the rehabilitation options for patients with severe to profound hearing loss are traditional hearing aids or cochlear implants (CI). A CI is a device which electrically stimulates cochlear nerve fibers. Enhancement of the capabilities of cochlear implant devices is an approach to the treatment (Shibata and Raphael, 2010). Although the current therapeutic possibilities are still limited, in some cases, such as in auditory neuropathy, a molecular diagnosis is worthwhile. It is estimated that approximately 40% of the auditory neuropathy cases have an underlying genetic basis (Manchaiah et al., 2011). In auditory neuropathy the function of the outer hair cells is normal, but the inner hair cell and/or the auditory nerve function is disrupted. Cochlear implantation in children with auditory neuropathy shows mixed results (Mason et al., 2003; Teagle et al., 2010). CIs bypass the cochlear sensory cells but are of limited use when the lesion is further upstream on the afferent auditory pathway. To overcome this problem, Wise et al. injected adenoviral vectors with neurotrophins in order to stimulate a resprouting of the auditory peripheral fibers required for the electrical stimulation of spiral ganglion neurons (Wise et al., 2010).

3 COCHLEAR GENE THERAPY

The methods of cochlear gene therapy are at present under energetic development. At the moment the only efficient way of treating hearing disorder originating from the inner ear is rehabilitation with traditional hearings aids or cochlear implantation (Lalwani et al., 2002a,b). Cochlear gene delivery methods are developed not only for future therapeutic methods of hearing improvement but also for the study of the molecular basis of deafness which is poorly known. Before cochlear gene therapy can be used in humans, it is necessary to develop transfer methods which maintain both the hearing function and the cochlear architecture (Lalwani et al., 2002a,b). Different viral vectors, plasmids, liposomes, and nanoparticles have been used as vectors in cochlear gene therapy studies (Chen et al., 2010; Kesser and Lalwani, 2009). Recent attempts have focused on the use of various stem cell types for repairing the auditory system (Kesser and Lalwani, 2009).

3.1 VIRAL VECTORS

The work of many research groups has resulted in a generation of number of viral vectors for various applications in gene therapy. Adenovirus, retrovirus, adeno-associated virus (AAV), and herpes virus-derived vectors have been tested for purposes of cochlear gene transfer in both *in vitro* and *in vivo* experiments. Each viral vector possesses its own characteristics, with consequences for its uses in different applications.

A number of routes of vector delivery have been established. The delivery methods include osmotic minipump infusion or microinjection into the scala tympani through the RWM (Aarnisalo et al., 2007; Derby et al., 1999; Jero et al., 2001a; Komeda et al., 1999; Pietola et al., 2008, 2012; Raphael et al., 1996; Yagi et al., 1999), infusion or injection into the scala tympani through a cochleostomy (Carvalho and Lalwani, 1999; Han et al., 1999; Lalwani et al., 1996, 1997, 1998a,b; Wareing et al., 1999), injection into the endolymphatic sac (Yamasoba et al., 1999), injection into the utricle (Praetorius et al., 2002), and application of gelatin soaked with the therapeutic agent to the RWM (Aarnisalo et al., 2006; Jero et al., 2001a; Wang et al., 2011). All other forms of vector delivery except the gelatin application involve damage to the inner ear structures, which increases the risk of hearing impairment due to a trauma to the cochlea (Lalwani et al., 2002a,b).

3.1.1 Adenovirus

Adenoviruses were isolated from human adenoid tissue in 1953 (Rowe et al. 1953). Since then, 51 different serotypes of adenoviruses among 6 different species have been identified. Classification is based on serological tests, the virus's ability to hemagglutinate red blood cells of rhesus monkey or rat, on restriction, and on sequence analysis. Most human adenoviruses do not cause infections in animals.

Adenoviruses are non-enveloped, icosahedral viruses. A linear, double-stranded DNA genome is enclosed in a capsid which is composed of 240 hexon capsomers and 12 penton capsomers with spike-shaped protrusions. The capsid also contains some hexon- and penton-base-associated proteins. Histon-like viral core proteins are responsible for the DNA's packaging.

The primary virus attachment to the host cell coxsackie- and adenovirus-receptor (CAR) is usually mediated by the terminal globular domain in the virus capsid (Bergelson et al., 1999), but also heparan sulfate proteoglycans have been shown to promote adenoviral attachment to some cell types (Dechecchi et al., 2001). The two common ways of viral entry after the binding to the host cell are clathrin- and caveolae/lipid raft-mediated endocytosis. The endosomal uptake of the virus is followed by stepwise dismantling of the capsid, which leads to microtubule-assisted transport and delivery of the core protein-coated viral genome to the nucleus of the host cell (Greber, et al., 1993). The first gene to be expressed is E1A which encodes a transactivator for the early genes for transcription (E1B, E2A, E2B, E3 and E4). The E2 region encodes proteins needed in the DNA replication (DNA polymerase, DNA-binding protein, and precursor of the terminal protein). The virion assembly in the nucleus starts approximately 8 hours after the infection and leads to the production of 10^4-10^5 particles per cell. The new viruses can be released after a proteolytic maturation by cell lysis 30-40 hours after the infection.

3.1.2 Adeno-associated virus (AAV)

The human adeno-associated virus was discovered as a contaminant in an adenovirus preparate (Castro et al., 1967). AAV belongs to the *Parvoviridae* family, in a separate *Dependovirus* genus because AAV needs a co-infecting helper virus for a productive infection. Even though AAV is very common in the human population (approximately 80% of humans are seropositive for AAV2) it has not been linked to any human illness.

AAV is a small, non-enveloped virus with an icosahedral capsid. It has a small (approximately 4.7 kilobases), linear single-stranded DNA genome. AAV2 DNA has inverted terminal repeats (ITR) which participate in the synthesis of leading-strand and double-stranded replicative intermediates. AAV does not encode a polymerase enzyme; it rather uses the polymerase of the host cell to replicate its DNA (Ni et al.,

1998). Between the ITRs are *rep* and *cap* genes. The *rep* gene encodes nonstructural proteins needed in replication and the *cap* gene encodes structural proteins needed in capsid formation.

AAV attaches to the host cells by binding to membrane-associated heparin sulphate proteoglycans (Summerford and Samulski, 1998). The internalization of the virus is helped by co-receptors (Gonçalves, 2005). The trafficking of AAV inside the host cell is not fully understood. Once AAV has entered the host cell nucleus, it can follow either the lytic or the lysogenic pathway. The lytic pathway develops in cells which are co-infected with a helper virus (for example Ad or herpes simplex virus). The lysogenic pathway develops in host cells in the absence of helper viruses. When AAV infects a host cell without a helper virus it enters into a latent state, its gene functions are suppressed and its genome integrates into the host cell's genome. The latently infected cell can be super-infected with a helper-virus whereupon the AAV gene expression machinery is activated. The provirus DNA dissociates from the host genome and is replicated and packaged into virions. Helper virus-induced cell lysis releases newly formed AAV virions from the host cell (Gonçalves, 2005).

AAV has at least 10 different serotypes. Even though different serotypes show a high amino acid homology, there are discernible differences in their functions. AAV serotypes 1, 2, and 3 need heparan sulfate proteoglycans as co-receptors for viral entry, whereas serotypes 4 and 5 do not use these co-receptors. AAV serotype 5 is shown to bind to siliac acid on the target cell's surface (Walters et al., 2001). These properties have to be considered when trying to use AAVs in targeted gene transfer. The effects of different serotypes on transduction efficiency in the cochlea have been studied by several groups (Bedrosian et al., 2006; Liu et al., 2007; Luebke et al., 2009; Stone et al., 2005). Liu et al. reported that serotypes 1, 2, 3, 5, 7, and 8 transduced only IHCs (Liu et al., 2005). Bedrosian et al. reported a transduction of HCs with serotype 1 and 8 after injection into newborn mouse pups (Bedrosian et al., 2006). Mosaic AAVs, which have both serotype 1 and 2 capsids, transduce IHCs in adult mice (Luebke et al., 2009). Conventional AAV vectors contain single-stranded DNA, which will be converted into double-stranded DNA before gene expression (Ferrari et al., 1996). Any AAV genome that reaches the nucleus will still require the synthesis of a complementary strand in order to achieve gene expression. This critical step can be effectively bypassed through the use of self-complementary AAV vectors, which achieve faster and stronger transgene expression than conventional AAV vectors (Yokoi et al., 2007).

3.1.3 Lentiviruses

Lentiviruses belong to the *Retroviridae* family and the lentivirus genus is divided into 6 subgenera: bovine, equine, feline, ovine/caprine, primate, and

unclassified. The primate lentivirus subgenus contains 3 different species: human immunodeficiency virus 1 (HIV-1), human immunodeficiency virus 2 (HIV-2), and simian immunodeficiency virus (SIV).

Lentiviruses contain an inner core in which the viral genome and also enzymes needed for the initiation of viral replication are situated (Haseltine, 1991). The core is surrounded by proteins which form a capsid around the core. The viral envelope, i.e. the outer membrane of the virus which is obtained from the previous host, consists of a lipid membrane (Haseltine, 1991; Varmus, 1988). Viral glycoproteins, which participate in the entry of the virus into a new host cell, are found on the surface of the envelope (Haseltine, 1991).

The lentivirus genome is composed of two single-stranded, polyadenylated RNA molecules, and the size of the genome is 9000 to 10000 base pairs (bp) (Clements and Narayan, 1981). There are three structural genes in the lentivirus genome: *gag* codes for a group-specific antigen, *pol* codes for polymerase, and *env* codes for the envelope. Lentiviruses also contain small open reading frames (ORFs) between the *pol* and *env* genes (Pyper et al., 1986; Zheng et al., 2005). The ORFs code for two regulatory proteins and four accessory proteins (Ratner et al., 1985; Zheng et al., 2005). The regulatory proteins are the transcriptional protein (Tat) and the regulator of virion gene expression (Rev). The accessory proteins are the "negative effector" (Nef), the viral infectivity factor (Vif) and the viral proteins r (Vpr) and u (Vpu). Long terminal repeats (LTRs) are located at both ends of the RNA strand. The LTRs are composed of U3, R and U5 regions. The U3 region contains important viral enhancer/promoter elements as well as regulatory elements (Narayan and Clements, 1989). The R region at both ends of the RNA strand contains a cap site from which transcription of the RNA strand is initiated, also the polyadenylation site which creates the poly(A) tail to the developing RNA strand, and the termination signal for viral RNA transcription (Hess et al., 1986; Guntaka, 1993).

Lentivirus infection begins by a high-affinity binding reaction between the surface glycoproteins on the viral envelope and CD4 molecules on a host cell (Haseltine, 1991). The virus enters the host cell by membrane fusion mediated by the viral envelope glycoproteins (Haseltine, 1991). Once the virus has entered the host cell's cytoplasm, its RNA is converted into DNA with the help of virus-specified RNA-dependent DNA polymerase, which is also known as reverse transcriptase, and ribonuclease (Haseltine, 1991). The formed DNA is called a provirus and it is integrated into the host genome by a viral enzyme integrase (Haseltine, 1991). The viral DNA stays permanently in the host cell genome. The virus rarely kills the host cell and the host cells are infected for life (Haseltine, 1991). Lentiviruses utilize, like other retroviruses, the host cell machinery to transcribe viral DNA into genomic RNA and messenger RNA (mRNA) (Guntaka, 1993; Narayan and Clements, 1989). The newly formed pre-mRNA is processed by polyadenylation and splicing (Narayan and Clements, 1989). Single viral mRNA is spliced into 46 different products (Purcell

and Martin, 1993). Once the viral proteins are fully processed they are assembled into virions in the cellular membrane (Nguyen and Hildreth, 2000).

3.2 HAIR CELL MANIPULATION

Hair cells in the organ of Corti are terminally differentiated and the maintenance of their post-mitotic state is essential for hair cell survival. Most of the cochlear cells in mice reach their post-mitotic state by E14.5 (Morsli et al., 1998). Almost all cell divisions in the developing auditory epithelium of mammals and birds stop before birth, and the maintenance of this state requires cyclin kinase inhibitors (CKIs) (Harper, 2001). CKIs are divided into two families, the Cip/Kip family which includes $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, and the Ink4 family which includes $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$. At least $p21^{Cip1}$, $p27^{Kip1}$, and $p19^{Ink4d}$ regulate cell cycle re-entry in the cells of the auditory epithelium. Co-deletion of $p19^{Ink4d}$ and $p21^{Cip1}$ leads in mice of an early postnatal stage in hair cells to cell cycle re-entry and a formation of supernumerary hair cells. An onset of abnormal proliferation in the auditory epithelium leads eventually to a loss of hair cells (Laine et al., 2007). $p27^{Kip1}$ has an important role in the developing and also the mature inner ear. $p27^{Kip1}$ is expressed in cochlear supporting cells but not in hair cells. In the supporting cells, $p27^{Kip1}$ acts as a negative regulator of the G1-S transition in the cell cycle (Chen and Segil, 1999; Harper, 2001; Löwenheim et al., 1999). Disruption of the $p27^{Kip1}$ gene in a knockout mouse model promotes cell proliferation in the organ of Corti of both postnatal and adult mice. The deletion of $p27^{Kip1}$ affects the morphology of these sensory cells, and all $p27^{Kip1}$ knockout mice show severe hearing impairment (Löwenheim et al., 1999). The role of different combinations of CKIs in the auditory epithelium is only poorly understood. While $p27^{Kip1}$ is required for the maintenance of the post-mitotic state of the supporting cells, other CKIs such as $p19^{Ink4d}$, $p21^{Cip1}$ and the retinoblastoma tumor suppressor protein (pRb) are acitve in hair cells (Chen et al., 2003; Mantela et al., 2005; Sage et al., 2005). pRb is the primary protein involved in cell cycle regulation in hair cells, and its function is to repress transcription of the genes required for G1-S transition. It is known that an inactivation of pRb family members in neurons makes these re-enter the S-phase. This cell cycle re-entry results in abnormal DNA replication, which is followed by cell death. p53 is a tumor suppressor gene which has a major role in DNA damage-induced cell death. p53 is activated when a cell's DNA repair system fails, and it upregulates Bax, a Bcl-2 family member which takes part in cell death regulation. p53 is also known to be involved in the initiation of cell death in cochlear and vestibular hair cells (Cheng et al., 2005).

Many studies in the field of inner ear therapy have focused on the regeneration of hair cells (Collado et al., 2008). The ability of avians to regenerate hair cells

(Corwin and Cotanche, 1988; Ryals and Rubel, 1988) has encouraged scientists to study the features which make this capacity possible. Elucidation of the mechanism of hair cell regeneration may lead to the preservation of the auditory function in hearing-impaired patients. Recent advances in hearing therapeutics have also taken advantage of stem cells. Embryonic stem cells are pluripotent and the aim is to differentiate these cells into hair cell-like structures (Coleman et al., 2007; Oshima et al., 2010). In the ideal case embryonic stem cells will be differentiated into fully functional cochlear hair cells and be used for a cell-based treatment of hearing loss.

AIMS OF THE STUDY

1. To evaluate the benefits of cochlear implantation on Usher syndrome III patients

2. To test the feasibility of gene delivery through the intact round window membrane in an *in vitro* model

3. To test the efficiency and safety of lentivirus vectors for inner ear gene transfer

4. To study the effects of adeno-associated virus vector-delivered shRNAs on the aminoglycoside damaged cochlea

5. To evaluate the effects of adeno-associated virus vector-delivered anti-clarin-ribozyme on the cochlea

MATERIAL AND METHODS

1. SUBJECTS AND SAMPLES

1.1 CONSENT AND ETHICS COMMITTEE PERMISSIONS (I)

USH3 patients and their family members were informed of the aims of the study. All participants signed a consent form. Research permissions were obtained from the Ethics committees of the participating hospitals.

1.2 USH3 PATIENTS (I)

Altogether 19 Finnish USH3 patients participated in the study. All patients received a cochlear implant during the years 1995 to 2005. The mean age of the patients was 47±19 years (youngest 12 years and oldest 72 years); seven of the patients were male and twelve female. Saliva samples for the mutation analysis were collected from 15 patients; the remaining four patients already had a USH3 diagnosis.

1.3 PATIENT DATA AND QUESTIONNAIRES (I)

A set of three questionnaires was sent to the patients (GBI, GHSI and a questionnaire designed for this study). Implantation data, audiometric data, imaging data, and data of the ophthalmologic examinations and vestibular tests of each patient were collected from medical records.

The GBI is a questionnaire developed to assess the patient's health status after an otorhinolaryngological intervention. The GBI questionnaire consists of 18 questions. The responses to all of the questions are averaged, so that all questions carry equal weight. The average score is transposed onto a benefit scale ranging from -100 (maximal negative benefit) through 0 (no benefit) to +100 (maximal benefit). The response to each question is placed on a five-point Likert scale ranging from a large deterioration to a large improvement in health status. The GBI questionnaire produces total score and three subscales: a general subscale, a social support subscale, and a physical health subscale.

The validated GHSI form contains 18 health status questions. These questions evaluate how the health problem has affected the patient's quality of life at the time the GHSI is completed. The response to each question is evaluated on a five-point Likert scale ranging from high to low health status. GHSI questionnaire also produces total score and three subscales: a general subscale, a social support subscale and a physical health subscale.

The questionnaire designed for the present study collected data about the implantation, about problems encountered with the use of the cochlear implant, previous use of hearing aids, previous and current status of vision, and vision-related symptoms. The responses to the questions were evaluated on a five-point Likert scale ranging from 1 (poor performance) to 5 (good performance).

Audiometric tests including pure-tone thresholds, speech reception threshold and speech recognition were conducted according to ISO standards. The change of pure-tone thresholds, calculated over 0.5, 1, 2 and 4 kHz, was derived from audiometric measurements made 5-10 years and one year prior to the surgery and 6-12 months after the surgery. Word recognition values were obtained from all patients by using bisyllabic, phonetically balanced words of the Finnish language validated for adults. The status of the patients' vision before and after the implantation was evaluated by four different questions in the questionnaire and by data collected from patient records.

1.4 ANIMALS AND ETHICS COMMITTEE PERMISSION (II-V)

Wild type CD-1 mice were used in the original the publications II-V. Animal care and experimental procedures were approved by the Local Ethics Committee for Animal Experiments, University of Helsinki, and the National Animal Experiment Board in Finland. The animal experiments were conducted in accordance with the European Convention guidelines.

1.5 CELL LINES (III, IV)

HeLa cell (American Type Culture Collection (ATCC) number CCL-2) cultures were used in the lentivirus experiments (**III**). Testing of the shRNA constructs was done in African green monkey kidney cell (COS) cultures (**IV**).

1.6 VIRAL VECTORS (II-V)

Adenovirus vectors, adeno-associated virus vectors and lentivirus vectors were used. A summary over all the viral vectors, their promoters, transgenes, and titers are presented in Table 1. These vectors were used in original the publications **II-V**.

Table 1. Viral vectors

Vector	Promoter	Transgene	Titer p/ml
Ad5	EFS	GFP	2x10^9
HOX (lentivirus)	EFS	GFP	8.5 x 10^6
WOX (lentivirus)	EFS	GFP	8.6 x 10^7
AAV2/2	CBA	Rz	3.2x10^13
AAV2/2	CBA	GFP	7,21x10^12
AAV2/2	U6	shRNA	1x10^12
AAV2/2	CMV	GFP	1x10^12

Ad5: adenovirus, serotype 5; EFS: human elongation factor 1-α minimal promoter; GFP: green fluorescent protein; AAV: adeno-associated virus (serotype 2/2); CBA: chicken β-actin promoter; Rz: ribozyme; shRNA: short hairpin RNA (p27 Kip1, p53, p27 Kip1+p53); CMV: cytomegalovirus promoter.
Titer particles/ml (p/ml)

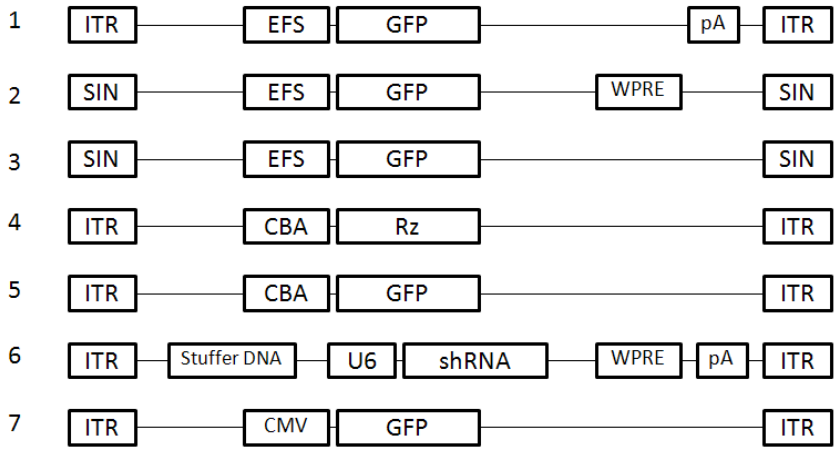


Figure 5. Schematic presentation of the viral vectors used in original publications II-V.

1. Adenovirus, type 5 (II)
2. HOX-GFP, lentivirus (III)
3. WOX-GFP, lentivirus (III)
4. AAV2/2-Rz (V)
5. AAV2/2-GFP (V)
6. AAV2/2-shRNA (IV)
7. AAV2/2-GFP (IV)

ITR: inverted terminal repeat; SIN: self-inactivating long terminal repeat; EFS: human elongation factor 1-α minimal promoter; CMV: cytomegalovirus promoter; CBA: chicken β-actin promoter; GFP: green fluorescent protein; Rz: ribozyme; shRNA: short hairpin RNA; WPRE: Woodchuck hepatitis virus post-transcriptional element; pA: poly adenylation site

2. METHODS

2.1 MUTATION ANALYSIS (I)

DNA from USH3 patients was obtained from saliva samples and extracted with Oragene™ kits (DNA Genotek Inc., Ontario, Canada). After DNA extraction, the exons of the *CLRN1* gene (NM_174878) were amplified with exon specific primers (Isosomppi et al., 2009) and Amplitaq® DNA Polymerase, and then purified with Exo-SAP (USB, Cleveland, OH, USA). The sequencing reactions were performed with an ABI3730 Automatic DNA sequencer using the ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

2.2 *IN VITRO* MODEL (II)

Cochleae from 30 CD-1 mice were removed and placed into DMEM culture medium. The RWM with the intact bony niche was removed under a microscope with a diamond micro drill. Detached RWMs were with epoxy glue fixed on a petri dish covering a 1mm hole in the dish. A plastic tube was fixed to the dish's hind side with adhesive. This tube was filled with 500µl of PBS. Hyaluronic acid ester (Merogel®, Medtronic Xomed) was used as the sponge material.

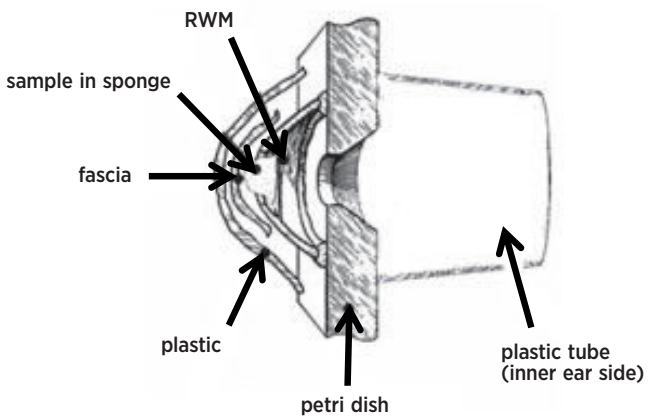


Figure 6. Schematic presentation of the RWM ***in vitro*** model

Redrawn from Aarnisalo et al., 2006

Ten RWMs were treated with $AgNO_3$, trichloracetic acid, or histamine-glycerol, and prepared for histological analysis. Samples were fixed with 4%PFA o/n, decalcified in 0.5M EDTA for 7-10 days, mounted to paraffin and sectioned. The samples were stained with hematoxylin-eosin and analyzed under light microscope. Samples of phosphate buffered saline (PBS) were collected at different time points from the plastic tube (20µl / sample). The absorbance of toluidine blue was evaluated spectrophotometrically (590 nm; Multiscan RC, Labsystems).

2.3 shRNAs (IV)

Four different pairs of $p27^{Kip1}$ sequences were designed from Genbank BC014296 and NM_009875 sequences and synthesized (Oligomer, Finland). The pairs were annealed and the generated double-stranded DNA fragments were cloned into the pSilencer 1.0-U6 plasmid (Ambion, USA) between ApaI and EcoRI under the U6-promoter. The p53 shRNA sequences were designed from GenBank: AF051368 and synthesized (Oligomer). The annealed fragment was cloned in pSilencer 1.0-U6. The created shRNAs were cloned into the AAV-vector either alone ($p27^{Kip1}$ or p53) or together ($p27^{Kip1}$ and p53).

2.4 PCR (II)

PCR was used for studying the permeability of the detached RMWs for adenoviral vector. Five µl of sample was used as template along with 5pmol of forward and reverse primers complementary to the GFP transgene. The samples were amplified with a PTC-100 thermal cycler using a reaction volume of 20µl. The 408 bp PCR product was visualized in 1% agarose gel and its concentration was determined semiquantitatively by Kodak Image Station 440CF analyzer.

2.5 RIBOZYME (V)

Three hammerhead ribozymes were designed to specifically recognize and cleave wild-type mouse clarin-1 mRNA. The ribozymes and the corresponding clarin-1 RNA oligonucleotide 12-mer substrates were synthesized by Dharmacon (USA). Cleavage time course reactions of the hammerhead ribozymes were performed under substrate excess conditions at 37°C in 40 mM Tris–HCl, at pH 7.5 and in the presence of either 20 mM $MgCl_2$ or 2 mM $MgCl_2$. The 12- mer substrates were 5'-end labelled with gamma ^{32}P-ATP resulting in 7-mer cleavage products.

2.6 TRANSDUCTIONS IN CELL LINES (III,IV)

HeLa cells were cultured on 13 mm cover slips (Menzel-Gläser, Braunschweig, Germany) on 24-well plates (NuncTM, Denmark) in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, USA) supplemented with 5% FCS (fetal calf serum) (PromoCell, Germany), GIBCO™ GlutaMAX™ –I Supplement (100x, L-Alanyl-L-Glutamine) (Invitrogen, USA), and Penicillin-Streptomycin (100x) (Sigma-Aldrich, Germany). The cells were transduced with HOX-GFP and WOX-GFP, respectively, as follows: 30 000 cells with 1 µl of vector, 40 000 cells with 5 µl of vector and 50 000 cells with 1 µl of vector. The vectors were gently added onto the growth medium with a pipette. The cells were cultured for three, five, and seven days at +37°C in a 5% CO_2 environment. The growth medium was removed and the cells were rinsed with PBS (2 x 600 µl). The cells were fixed in 4% PFA (paraformaldehyde, Sigma-Aldrich, USA) in PBS for 5 min, rinsed again in PBS, and mounted in Vectashield® Mounting Medium for fluorescence imaging (Vector Laboratories, USA). (**III**)

The silencing effect of the different shRNAs was tested by transfecting 1 µg of shRNA-plasmid into COS cells in culture with Lipofectamine or Lipofectamine 2000 (Invitrogen, USA) reagents according to the manufacturer's instructions. Since proliferating COS cells do not express $p27^{Kip1}$, we cotransfected 1µg of $p27^{Kip1}$ cDNA expressing vector together with the shRNA plasmids. Bluescript plasmid (Stratagene, USA) was used as a control. The expression levels of $p27^{Kip1}$ in COS cells were analyzed from 10 µg of protein from cell lysates with 12% SDS-PAGE acrylamide gel-electrophoresis and Western blotting using Hybond-C Extra membrane (Pharmacia, USA) and rabbit polyclonal anti-$p27^{Kip1}$ antibody (1:1000; Santa Cruz, USA) together with Anti-rabbit Ig-HRP visualized by ECL Western blotting substrate (Pierce, USA). The best silencing sequences (shRNAs 2 and 3) were with their respective U6 promoters digested out from the pSilencer 1.0-U6 with BamHI and cloned next to each other into the AAV-vector (AAV2/2) in front of the woodchuck hepatitis virus post-transcriptional element (WPRE). The CMV promoter present in the AAV-CMV-WPRE construct was removed with EcoRI and XhoI, and uncoding stuffer DNA was cloned instead to keep the viral DNA size optimal. As controls for Western blotting, we were growing mouse embryonic fibroblasts (MEF) from p53 targeted embryos (p53-/- and p53+/-, Jackson laboratory, USA). Western blot analysis was performed as above with anti-p53 antibody (1:300; sc-6243 Santa Cruz). (**IV**)

2.7 ANIMAL SURGERY (II-V)

Mice were anesthetized with an i.p. injection of anesthetic (Table 2). Exposure of the cranial base and middle ear bulla was performed through a ventral paramedian incision as described previously by Jero et al. (2001b). The vectors were injected

slowly into the inner ear by a glass capillary (inner diameter 0.5 mm, SM100F-15, Harvard apparatus, tip diameter 5 µm) with the help of a microinjector (Narishige, Japan) and a preparation microscope (Olympus SZX12). After the injection, the RWM was sealed with a small piece of thin fascia and the incision was closed in layers. The total operating time was approximately 15-20 min.

Table 2. Anesthetics

II, III	Midazolam 2mg/kg (Midazolam Hamelm 5mg/ml)	Ketamine 100mg/kg (Ketalar® 50mg/ml)
IV	Xylazine 10mg/kg (Rompun® 20mg/ml)	Ketamine 100mg/kg (Ketalar® 50mg/ml)
V	Medetomine 1mg/kg (Domitor® 1mg/ml)	Ketamine 75mg/kg (Ketalar® 50mg/ml)

2.8 *IN VIVO* TRANSDUCTIONS (III-V)

24 white, 11- to 13- week-old male CD-1 mice (weight approx. 30 g, Harlan, Netherlands) were used in the lentivirus experiment. The mice were divided into two groups: a kanamycin group and an untreated group. The animals in the kanamycin group were pretreated with kanamycin injections 15 days prior to the surgery; the other group received no pretreatment. Each animal in the pretreated group received daily 0.6 ml of kanamycin sulfate (0.02 ml/g daily, 2 x 0.3 ml/day i.p., 400 mg/kg, Sigma-Aldrich, USA). In addition, both of these two groups were subdivided into three separate groups: HOX-GFP, WOX-GFP, and saline control. A total of six groups were formed with four mice in each group.

One µl of pure lentivirus vector was introduced into the inner ear of the mice by direct microinjection through the round window membrane (**III**).

20 white, 11- to 13- week-old male CD-1 mice (weight approx. 30 g, Harlan, Netherlands) were used in the shRNA experiment. The mice were divided into groups according to which AAV-shRNA vector they received: $p27^{Kip1}$ (AAV-p27-shRNA), p53 (AAV-p53-shRNA), or $p27^{Kip1}$+p53 (AAV-p27+p53-shRNA). The fourth group received only saline injections. These four groups (16 mice) were pretreated with kanamycin injections 15 days prior to the surgery. Each animal received 0.6 ml of kanamycin sulfate as described earlier. The fifth group received AAV-EGFP injections without kanamycin pretreatment. AAV-shRNA together with AAV-EGFP 0.5 + 0.5 µl, or 1 µl of saline was introduced into the inner ear of the mice by injecting directly through the round window membrane (**IV**).

40 white, 11- to 13-week-old male CD-1 mice (weight approx. 30 g, Harlan, Netherlands) were used in the ribozyme experiment. The animals were divided into three groups (AAV-GFP, AAV-Rz, and saline) and there were four animals in

each group. 1 µl of virus vector (AAV-GFP or AAV-Rz) or saline was injected into the inner ear through the round window membrane. Those animals in whom the stapedial artery ruptured during the procedure were excluded from the study (**V**).

2.9 TISSUE PROCESSING (II-V)

In the further course of the experiment, the animals were sacrificed as follows: lentivirus-injected animals 14 days after the surgery (**III**) and AAV injected animals one week (**V**) or one month (**IV**,**V**) after the surgery. All animals received an i.p. overdose of sodium pentobarbital (0.3 mg/10 g, Mebunat, Orion, Finland). After losing consciousness, the animals were perfused transcardially through the left ventricle with 20 ml of saline or PBS followed by 10 ml of 4% PFA. The inner ears (cochleae), and in some cases selected internal organs (brain, liver, heart, lungs, bladder, kidney, spleen, part of the duodenum, femoral bone marrow), were harvested and postfixed in 4% PFA overnight at +4°C. The cochleae were decalcified in 0.5 M EDTA for 7-14 days and rinsed afterwards in PBS. All the internal organs were rinsed several times in PBS and left in PBS overnight at +4°C. The brains were immersed in 20% sucrose in PBS (sucrose, Riedel-de Haën, Germany) and left overnight at +4°C. The decalcified cochleae were mounted in paraffin (**III-V**) as also all the internal organs (**III**). The paraffin-embedded samples were cut into 5µm sections.

2.10 SAMPLE ANALYSIS (II-V)

Ten RWMs with the bony niche attached were prepared for histological analysis. The samples were treated accordingly (control, $AgNO_3$ (5 and 20 %), trichloroacetic acid, or histamine-glycerol) and after the treatment washed with PBS for 10 min. The samples were fixed with 4% PFA overnight at 4°C, and then embedded in paraffin, sectioned (5µm), deparaffinized and stained with hematoxylin-eosin (Sigma). The slides were mounted with Permount (Fisher Scientific) (**II**).

All PFA-fixed, paraffin-embedded tissue sections (cochleae and organs) were deparaffinized according to the following protocol: rinse in xylene (Sigma-Aldrich) 2 x 10 min, hydration through a downward-graded series of EtOH (96%, 85%, 70%, 50%, 30%) for 2 min each with a final rinse in PBS 3 x 5 min.

The GFP expression was detected from paraffin-embedded cochlear tissue sections and paraffin-embedded internal organs. The deparaffinized tissue sections were mounted in fluorescent Vectashield® Mounting Medium (Vector Laboratories, USA) under glass coverslips (**III-IV**).

The antigen retrieval was performed by incubating the sections for 20 min at room temperature in proteinase K solution [10 µg/ml (Roche Applied Science) + 10 mM Tris-HCl, pH 7.6] (**III-IV**).

CD4 and CD8-β staining: The sections were rinsed in PBS-T (Triton X-100, Fluka, Switzerland) 2 x 5 min and stained with rat monoclonal CD4 and CD8-β antibodies (2 µg/ml, Santa Cruz Biotechnology, Inc., USA) diluted in PBS-T with 1% NRS (normal rabbit serum, from ABC kit). The sections were incubated with primary antibody for 24 hours in a moist chamber at +4°C. The bound antibody was visualized with a Vectastain® Elite ABC kit (Vector Laboratories, USA) according to the manufacturer's instructions. DAB (diaminobenzidine tetrahydrochloride, Sigma-Aldrich, USA) was used according to the manufacturer's instructions for the localization of peroxidase in the tissue sections. Finally, all the samples were mounted in Permount® (Fisher Scientific, USA) under glass coverslips. The inflammatory reaction caused by the vectors and also the amount of inflammatory cells on the analyzed tissue sections were only roughly estimated according to the following scale: mild/few inflammatory cells, moderate/some inflammatory cells, severe/many inflammatory cells. (**III**)

TUNEL-staining: Apoptosis of hair cells and of other inner ear cell types was detected with a fluorescence *in situ* cell death detection kit (Roche Applied Science, Germany), also called the TUNEL-staining method, according to the manufacturer's instructions. After staining, the slides were mounted in fluorescent Vectashield® Mounting Medium (Vector Laboratories, USA) under glass coverslips. (**III-V**)

2.11 FLUORESCENCE MICROSCOPY (II-V)

All samples were analyzed under an Axioplan 2 fluorescence microscope (Zeiss, Germany). The GFP fluorescence was analyzed with a FITC filter (fluorescein isothiocyanate), which was combined with a standard TRITC filter set to exclude autofluorescence. Digital images were analyzed with the Axiovision 3.0 program (Zeiss, Germany).

2.12 STATISTICAL ANALYSIS (I-V)

The data from the audiological records and questionnaires were analyzed using Prism 3.03 for Windows. The chi-square test, Pearson's correlation test and t-test were applied. P-values less than 0.05 were considered statistically significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) (**I**).

The absorbances of toluidine blue were statistically analyzed with GraphPad Prism software (t-test, Graphpad Software). (**II**)

All TUNEL positive nuclei were counted (mean ± SEM) and the results statistically analysed with Student's t-test (**III**-**V**) and ANOVA (**V**) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (**III**-**V**)

RESULTS AND DISCUSSION

1. EFFECT OF COCHLEAR IMPLANTATION ON THE QUALITY OF LIFE IN FINNISH USH3 PATIENTS (I)

1.1 USH3 PATIENTS

Usher syndrome type 3 is the most common form of Usher syndrome in Finland. The patients are afflicted with hearing loss, retinitis pigmentosa and in some cases variable vestibular dysfunctions. They are considered to have a profound sensory handicap. Cochlear implants are widely used among USH3 patients. Implanted USH1 patients have been studied previously (Liu et al., 2005; Pennings et al., 2006); however, this is the first study ever done on USH3 patients. Previous studies have shown that cochlear implantation in deaf-blind adults is as successful as in other prelingually and postlingually deaf adults (Loundon et al., 2003). The USH3 genotype of participating patients was determined from saliva samples. All 19 genotyped patients were of Finnish ancestry and were homozygous for the Finmajor mutation (p.Y176X). Although all of our patients had the same p.Y176X mutation in the *CLRN1* gene, the hearing, speech discrimination, and the change in the hearing ability varied significantly after implantation. The factors which cause the differences in the clinical phenotype and the prognosis of the syndrome are still unknown. A recent study by Ebermann et al. suggests that alternative splicing of the USH genes or effects of modifier genes could explain some of the differences in the clinical phenotype (Ebermann et al., 2010). Västinsalo et al. have studied alternative splicing of the *CLRN1* gene. Also they concluded that the structure of *CLRN1* is more complex than previously assumed and that multiple splice variants of *CLRN1* may explain the variation in the clinical phenotype among USH3 patients (Västinsalo et al., 2011).

All USH3 patients had used hearing aids prior to the cochlear implantation. Conventional hearing aids had been used for 30 ± 14 years and cochlear implants for 6 ± 4 years. The mean age of the patients was 41 ± 17 years (Table 3). The implant devices received by these patients came from two manufacturers: 7 patients got multichannel devices from MED-EL and 12 patients Nucleus multichannel devices from Cochlear.

CT and MRI scans were performed on all patients. All patients showed a normal morphology of temporal bone and inner ear. Prior to their surgery, the patients had also gone through electronystagmography including caloric tests. 14 patients had normal caloric responses, two patients had weak responses and in three patients the caloric responses were not evaluated preoperatively. The patients did not have clinical vestibular problems.

1.2 AUDIOLOGICAL RESULTS

The mean preoperative hearing threshold of our patients one year prior to the surgery was 110 ± 8 dBHL (PTA, pure tone average, 0.5-4kHz), and the aided hearing threshold at the same time 58 ± 11 dBHL (PTA 0.5-4kHz). The postoperative hearing threshold was significantly better: 34 ± 9 dBHL (PTA 0.5-4kHz) ($p < 0.01$, compared to preoperative aided PTA, t-test) (Table 4).

Speech recognition of the patients was tested with recorded Finnish bisyllabic words. The aided speech recognition scores demonstrated a progressive drop from 17 ± 25% (5-10 years prior to surgery) to 4 ± 9% (one year prior to surgery). The postoperative recognition score with implant (0.5-1.5 years after the surgery) was significantly better: 52 ± 33% ($p < 0.001$, compared to the preoperative (0-1 year) value, t-test). Similar results were seen with implanted USH1 children (Liu et al., 2008; Pennings et al., 2006). The most important predictors for the understanding of speech in postlingually deafened adults are the duration of the deafness and the residual hearing before implantation (Gantz et al., 1993; Van Dijk et al., 1999). Speech discrimination is significantly poorer in prelingually deafened CI recipients than in postlingually deafened patients. The prelingually deafened implant recipients are also known to have a higher rate of non-use of the implant (David et al., 2003; Waltzman et al., 1992). Patients who already use sign language as their primary mode of communication may even turn down the opportunity to receive a CI.

Table 3. USH3 patient information

USH3 patients n=19	Age (years)	Age at implantation (years)	Use of conventional HA (years)	Use of CI (years)
Mean±SD	47±19	41±17	30±14	6±4
Median	51	44	29	5
Min	12	10	12	1
Max	72	64	58	13

HA: hearing aid; CI: cochlear implant

Table 4. The preoperative and postoperative mean hearing thresholds of USH3 patients

	Preoperative (5-10 years)	Preoperative (1 year)	Preoperative with HA (1 year)	Postoperative with CI (1 year)
Hz	**dB**	**dB**	**dB**	**dB**
500	100±9	101±8	50±12	33±6
1000	105±7	110±8	49±12	35±8
2000	108±9	115±6	64±11	34±12
4000	105±14	114±9	69±9	33±11
PTA(0,5-4 kHz)	104±10	110±8	58±11	34±9

Hz: hertz; dB: decibel; PTA: pure tone average

1.3 QUESTIONNAIRES

The GBI is a questionnaire which assesses the patient's health status after an otorhinolaryngological intervention (Lassaletta et al., 2006; Robinson et al., 1996). The patient's quality of life (QOL) is by the GBI questionnaire measured in three categories: social, general, and physical. The mean GBI total score in Finnish USH3 patients was 30 ± 19 (median 31, min 3, max 61). The mean social subscore was 14 ± 18 and the mean physical subscore 0 ± 17. In our study the GBI total score was positive, which means that the patients felt that their health status had improved after the intervention.

The GBI results were plotted against age of the patient (r, 0.402), age at implantation (r, 0.427), or against speech recognition one year postoperatively (r, 0.38). In our results, neither the patients' age nor their age at implantation nor their speech discrimination one year after the implantation was showing any correlation with GBI results. The U.K. Cochlear Implant Study Group (CISG) had studied the benefits of cochlear implantation in 277 patients by means of the GBI (UK Cochlear Implant Study Group, 2004). We plotted our GBI total scores against the patients' age and compared the results with the U.K. CISG's results. Four USH3 patients out of 19 did not fit into two standard deviations of the mean benefit of 227 implanted patients of the U.K. CISG study. The visual scores were also evaluated: there was no significant difference between the current visual score and the visual score before the implantation. The GBI results show that USH3 patients benefit from the implantation as much as implanted patients with other deficits (Lassaletta et al., 2006). It is possible that our retrospective, cross sectional study design was causing bias in the results. USH3, however, is a rare syndrome and the number of USH3 patients is limited, and a prospective study would be impossible to execute.

The validated GHSI form comprises 18 health status questions which evaluate psychological, social, and emotional aspects of QOL at the time the GHSI is completed. The total score of the GHSI data of hearing (59 ± 9), when compared to that of vision (56 ± 14), was not significant (score range 0 to +100, $p > 0.05$).

Our self-designed questionnaire gathered information about the CI and its use. Most of the patients (15/17, 88%) reported that they used the CI all the time or 12-16 hours per day. Most patients (18/19, 95%) did not need any assistance in setting the CI. Over half of the patients (11/19, 58%) felt it easier or much easier to use a CI than conventional hearing aids. Most patients (11/18, 61%) did not experience tinnitus or did so only seldom. Those who reported tinnitus had noticed that the implant had an effect on tinnitus sound. 8 out of 13 (62%) patients reported that the cochlear implant either reduced or even stopped the tinnitus sound.

2. PERMEABILITY OF DETACHED INTACT RWM AN *IN VITRO* MODEL (II)

2.1 *IN VITRO* RWM MODEL

Previous *in vivo* studies have shown that the inner ear can be accessed with viral vectors through the RWM (Derby et al., 1999; Jero et al., 2001a; Komeda et al., 1999; Raphael et al., 1996; Yagi et al., 1999). The role of lymphatic vessels and/or blood vessels in the transmission of agents to the inner ear is difficult to assess, which prompted a need for an *in vitro* model for RWM gene transfer studies. We created such a model by attaching intact mouse RWM to a petri dish.

It is known that the coxsackievirus and adenovirus receptor (CAR) is responsible for the expansion of adenovirus infection by disrupting tight-junctions, this way letting the virus escape across epithelial barriers to the environment (Arnberg N, 2009; Cohen et al., 2001; Walters et al., 2002). Histamine disrupts tight-junction protein expression (Flynn et al., 2009; Zabner et al., 2003) and, therefore, may enhance Ad-mediated gene transfer into the cochlea through the RWM. In order to produce changes in the membrane, we treated the outer surface of the RWM with $AgNO_3$ (5 or 20%), trichloroacetic acid or histamine-glycerol. After treatment, some of the samples were sectioned and evaluated under light microscopy. Control samples showed a normal inner and outer surface of the RWM. After the $AgNO_3$ (5%) treatment, we observed destruction of the outer epithelial lining. After $AgNO_3$ (20%) treatment, all three layers of the RWM showed destruction. After the trichloracetic acid treatment, the outer epithelial surface was damaged. No changes could be seen under light microscopy after the histamine-glycerol treatment. This was surprising, since there are previous data which suggest that histamine causes changes to the RWM which lead to evident increase in permeability (Dennis et al., 1976).

2.2 PASSAGE THROUGH INTACT RWM

The role of the RWM in secretion and absorption has been known for many decades (Miriszlai and Benedeczky, 1978; Richardson et al., 1971). Since then, many animal studies have focused on the permeability of the RWM. It is known that even though the RWM is three-layered it behaves more like a semipermeable membrane. A number of agents penetrate the RWM, including antibiotics, antiseptics, arachidonic acid metabolites, local anesthetics, toxins, albumin, cationic ferritin, and horseradish-peroxidase. The permeability of the RWM is influenced by the agent's size, configuration, concentration, liposolubility, electric charge, and also

by the thickness of the membrane itself (Goycoolea et al., 1988; Goycoolea, 1992). As we wanted to change the permeability of the RWM, we treated it with $AgNO_3$, trichloracetic acid, or histamine-glycerol. Trichloracetic acid and histamine-glycerol reduced the permeability of the membrane, and the reduction was also significant with $AgNO_3$ at the concentrations of both 5% and 20%. Dennis et al. suggested that topical administration of histamine causes vasodilated edema in the middle ear mucosa and also an increase in the RWM's permeability (Dennis et al., 1976). Also inflammation in the middle ear mucosa is known to cause changes in RWM permeability; the permeability is increased at the beginning of the inflammation, but gradually decreases due to swelling of the RWM while the inflammation continues (Goycoolea, 1992). This was also seen in our results. Histamine is also known to disrupt tight-junction protein expression (Flynn et al., 2008; Zabner et al., 2003). The failure of the histamine-glycerol treatment to enhance the RWM's permeability might result from a recovery tight-junction protein expression in the RWM due to a wrongly chosen time window.

We chose to use toluidine blue in our RWM passage experiment because of the small size of its molecules; Juhn et al. had reported that small molecules are transported quickly through the RWM (Juhn et al. 1989). The absorbance of toluidine blue was measured spectrophotometrically from the PBS samples we took from the inner ear side of the RWM model. Toluidine blue passed through the pretreated ($AgNO_3$, trichloracetic acid, or histamine-glycerol) RWM in a time-dependent manner.

Also changes in osmolality can have consequences for the permeability of the RWM. Since the RWM is permeable to water, osmotically active substances in the middle ear can alter the fluid osmolality in the inner ear. This can result in membrane displacement (Juhn et al., 1988). To avoid this problem, both the Ad vector solution and the sample collection chamber contained physiological buffer. The passage of Ad vector through the intact RWM was studied at three different time points: one, three, and five days after virus administration. We detected Ad vectors from the samples with PCR. A previous study by Jero et al. suggested that Ad passes through intact RWMs *in vivo* (Jero et al., 2001a). Our results show that Ad passes through intact RWMs also *in vitro*. Viral transmission through the RWM increased during the first two days and stabilized at day three, i.e. the Ad vector's passage happened in a time-dependent manner. The passage of the Ad vector was slower than the passage of toluidine blue. This may result from the bigger molecular size of Ad vector compared to toluidine blue.

2.3 EXPRESSION OF GFP IN THE RWM

Evaluation of the expression of GFP was our second method of assessing the molecular transport through the RWM. The treated RWMs were removed from the petri dish at three different time points: after one, three, and five days. At day one the GFP expression was not yet detectable, but at days three and five the samples showed GFP expression in the RWM structures. GFP fluorescent cells were seen especially in the outer surface of the RWM. Even though Ad-GFP transduced cells of the RWM, the vectors also caused damage to the outer surface of the membrane. This may have inhibited further transfer of Ad-GFP through the membrane. Aside of this, it is known that first generation Ad vectors cause immune reactions in the inner ear (Jero et al., 2001a; Luebke et al., 2001b), while toxic effects can be avoided by deleting several viral genes from the vectors (Holt, 2002).

3. HOX AND WOX LENTIVIRUSES IN INNER EAR GENE TRANSFER (III)

3.1 TRANSDUCTION EFFICIENCY OF HOX-GFP AND WOX-GFP

Lentivirus vectors are based on the human immunodeficiency virus type 1 (HIV-1) and they have been studied intensely since 1996. Lentivirus vectors infect both dividing and nondividing cells, which makes them potential vectors for cochlear gene transfer. Lentivirus-mediated gene transfer is stable due to the integration of the vector genome into the target cell genome, by which the expression of the transgene is also long-term (Naldini et al., 1996).

We tested HOX and WOX lentivirus vectors first *in vitro* in HeLa cell culture. Both vectors transduced HeLa cells. We saw a clear GFP expression in transduced HeLa cells after three, five, and seven days (Figure 7A). The average percentage of transduced HeLa cells after 7 days of incubation was 66% ± 12%. Pellinen et al. transduced cancer cell lines successfully with vectors similar to HOX and WOX lentivirus vectors (Pellinen et al., 2004). A previous study by Han et al. showed that SGNs and glial cells can be transduced by lentivirus vectors in cochlear explants (Han et al., 1999). We studied the transduction efficiency of lentivirus vectors also *in vivo* in CD-1 mice. GFP transgene expression was restricted to the mesothelial cells in the perilymphatic space of the cochlea (Figure 7B). Delicate, but genuine GFP expression was observed in the cells surrounding the scala vestibuli and scala tympani while the organ of Corti and the tectorial membrane did not show any GFP expression. Han et al. were studying lentivirus vectors *in vivo* in a guinea pig cochlea. They saw GFP expression also in the perilymphatic parts of the cochlea after infusion with an osmotic minipump (Han et al., 1999). Differences in the localization of GFP expression may result from the promoters used for transgene expression. Han et al. used the CMV promoter, which may have influenced the localization of GFP in the cochlea. A previous study by Salmon et al. indicated that the choice of promoter affects the level of lentivirus vector-mediated transgene expression in human hematopoietic stem cells (Salmon et al., 2000). We detected GFP expression only in the cochlear sections of animals injected with the vector. Untreated contralateral control cochleae showed no GFP expression. It appears that lentivirus vectors are mainly localized in the injected ear.

We compared the transduction efficiencies of the two lentivirus vectors. We did not observe any differences in transduction efficiency between the two, even though the WOX-GFP vector has a WPRE expression enhancer element attached to it and WPRE is known to increase transgene expression (Pellinen et al., 2004; Zufferey et al., 1999). Cochlear sections from the saline injected control mice showed no

GFP expression. Altogether, the results suggest that lentivirus vectors can be used in delivering transgene products into the perilymph.

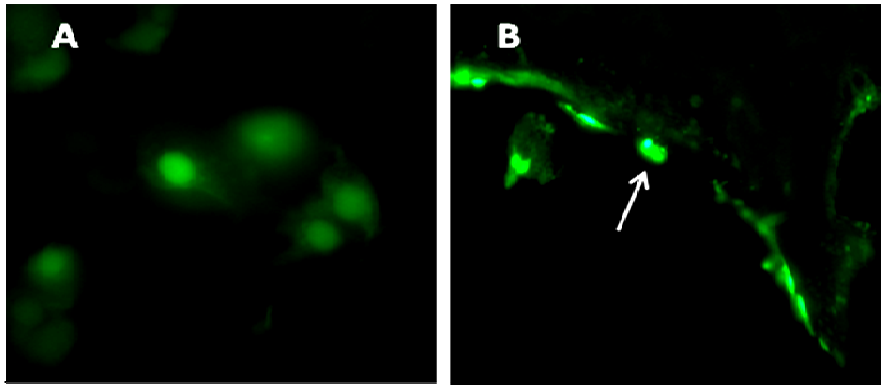


Figure 7. Lentivirus vector mediated GFP expression

A) HeLa cells

B) Lining cells of scala tympani (arrow)

3.2 TRANSDUCTION EFFICIENCY OF HOX-GFP AND WOX-GFP AFTER KANAMYCIN TREATMENT

We evaluated the effect of the aminoglycoside antibiotic kanamysin on transduction efficiency because it is known that aminoglycoside antibiotics damage primarily the OHCs while IHCs and SCs are less affected (Murillo-Cuesta et al., 2010; Rybak, 2007; Wu et al, 2001). Wu et al. and Murillo-Cuesta et al. were studying kanamycin ototoxity in CBA, C57B/6, and BALB mice (Murillo-Cuesta et al., 2010; Wu et al, 2001) but not in CD-1 mice. We observed, in our CD-1 mice, kanamysin-induced apoptosis in both the IHCs and the OHCs, which is in accordance with the previous results. We detected apoptotic cells also in the stria vascularis and in the structures of the vestibular system. Further, we studied whether damage caused by kanamycin has any effect on the transduction efficiency of HOX-GFP and WOX-GFP but we saw no difference in the pattern of GFP expression between the kanamycin treated and the control animals.

The number of apoptotic outer hair cells, inner hair cells and vestibular sensory cells per section was calculated and the results statistically analyzed. The number of apoptotic OHCs in the HOX-GFP and WOX-GFP group were significantly higher than in the saline group. The WOX-GFP group also showed a significantly higher number of apoptotic IHCs than the saline group (Table 5).

Table 5. Difference in the number of apoptotic hair cells in HOX, WOX, and saline groups (statistical significance $p < 0.05$)

cells	groups	P value ($p < 0.05$)
OHC	WOX vs saline	p = 0.0253
	HOX vs saline	p = 0.0486
IHC	WOX vs saline	p = 0.0211

3.3 DISTANT SPREAD OF HOX-GFP AND WOX-GFP TO PERIPHERAL ORGANS

We looked for a possible distant spread of lentivirus vectors from paraffin-embedded brain, liver, heart, lung, duodenum, kidney, bladder, spleen, and femoral bone marrow tissue sections. The sections were checked for GFP expression. The only expression we found was in the liver of one HOX-GFP injected animal; i.e. the expression was localized in the cells surrounding the portal vein. All other inspected tissues were free of GFP expression. Our results suggest that any distant spread of lentivirus vectors appears to be minimal.

3.4 IMMUNE RESPONSES AFTER TRANSDUCTION WITH LENTIVIRUS VECTORS

Inflammatory reactions in the inner ear can damage the sensory epithelia of the cochlea and also the vestibular system. We studied whether lentivirus vectors cause inflammatory reactions in the cochlea. CD4 and CD8-β antibodies will stain macrophages, T cells, and dendritic cells and were therefore chosen to be used in the experiment. Inflammatory reactions caused by other gene transfer vectors have already earlier been evaluated with CD4 and CD8-β antibodies (Staecker et al., 2001). It is known that adenovirus- and herpes virus-derived vectors will cause a significant inflammatory reaction in the cochlea at the site of vector injection. Any inflammatory reactions elsewhere in the cochlea and in the contralateral cochlea are milder (Staecker et al., 2001). In addition, the amount of inflammatory cells observed in the saline control animals was equivalent to the amount detected in vector treated animals (Staecker et al., 2001). The new second-generation adenovirus vectors seem to cause a milder immune response (Bessis et al., 2004). We found only few inflammatory cells in the cochlear sections from the vector injected ear. The lymphocytes were found in the perilymphatic space of the cochlea or in the vestibular system, but we did not find any lymphocytes in the endolymphatic space of the cochlea. The detected inflammatory response to the lentivirus vectors was mild (Table 6), and we suggest that the injection of the vector into the inner ear does not increase the inflammatory reaction in the cochlea. Single lymphocytes were also detected in the samples from the contralateral control ears. Few vector

injected animals had lymphocyte aggregates in the vestibular area. However, some inflammatory cells are always present in the inner ear structures. We conclude that lentivirus vectors do not cause any significant inflammatory response in the inner ear, and thus provide a safe method for inner ear gene therapy.

Table 6. Level of immunological reaction in the cochlea

Group	mouse #1	mouse #2	mouse #3	mouse #4
HOX	+	+	+	–
WOX	+	+	–	+
saline	+	+	–	+
HOX with kanamysin	+	–	+	+
WOX with kanamysin	–	+	+	+
saline with kanamysin	+	+	+	–

Scale: – no reaction, + mild reaction, ++ moderate reaction, +++ severe reaction

4. EFFECTS OF AAV-shRNA CONSTRUCTS ON KANAMYCIN DAMAGED COCHLEA (IV)

4.1 TESTING OF shRNA CONSTRUCTS IN COS CELLS

The functionality of shRNAs was tested in COS cell cultures. After 24 or 48 hours of incubation cells were collected and proteins extracted. In dividing COS cells no $p27^{Kip1}$ expression was detected so that inorder to test the $p27^{Kip1}$ constructs, $p27^{Kip1}$ cDNA was also co-transfected into COS cells. shRNAs were cloned into the pSilencer 1.0-U6 vector. Western blot analysis with anti-$p27^{Kip1}$ antibody showed that the best silencing was produced with $p27^{Kip1}$ shRNAs 2 and 3. These results suggest that the $p27^{Kip1}$ shRNAs 2 and 3 are most fitting to silence $p27^{Kip1}$ expression in cells. Therefore, these two $p27^{Kip1}$ shRNAs were first cloned together and then into the AAV vector (Paterna et al., 2000) to give rise to the AAV-p27-shRNA.

The p53 shRNA was tested by transfections into COS cells which express p53. In the Western blot analysis, proteins were also extracted from mouse embryonic fibroblasts (MEFs) which had been isolated from gene-targeted p53 null embryos ($p53^{-/-}$) and heterozygous embryos ($p53^{+/-}$). As expected, no p53 could be detected in $p53^{-/-}$ MEFs whereas $p53^{+/-}$ MEFs showed detectable levels of p53 expression. In COS cells the p53 expression had clearly decreased after 24 hours of transfection, and after 48 hours the level appeared nearly normal. This result suggests that the p53 shRNA can be used for silencing experiments in cells. The p53 shRNA fragment was cloned into the AAV vector either alone in order to give rise to the AAV-p53-shRNA, or together with the two $p27^{Kip1}$ shRNAs 2 and 3 to give rise to AAV-p27+p53-shRNA.

4.2 AAV-EGFP AND AAV-shRNA VECTOR EXPRESSION IN MOUSE COCHLEA

Functional shRNA constructs and AAV-EGFP were injected into kanamycin-damaged mouse cochleae through the intact RWM. The small packaging size (~4.7kb) of the AAV virus capsid made it impossible to combine shRNAs and EGFP into the same construct. Instead, the two constructs were mixed and injected at the same time. After a month, an EGFP expression was clearly detectable in the cochleae of all injected mice (Figure 8). The expression was limited to the organ of Corti and was seen in all cochlear turns. However, the expression was most intense at the site of the injection. Previous studies with AAV-GFP have shown similar results (Kilpatrick et al., 2011; Lalwani et al., 1998a,b). One control group of mice was injected with AAV-EGFP but did not receive kanamycin prior the injections. Kanamycin had no

effect on the expression pattern of the EGFP in the cochlea. These observations are in accordance with our previous results with lentivirus vectors (Pietola et al., 2008). The influence of shRNAs on the function of auditory epithelium was not studied. It is currently not known whether these shRNAs have an effect on hearing *in vivo*.

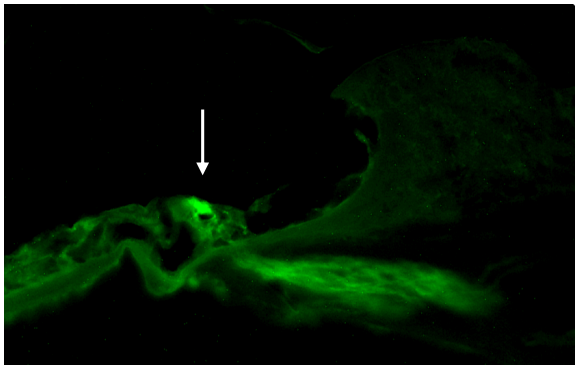


Figure 8. AAV-GFP expression in mouse cochlea.

AAV-GFP expression in the inner hair cell (arrow)

4.3 IMPROVED CELL SURVIVAL IN THE INNER EAR OF AAV-shRNA TRANSDUCED MICE AFTER AMINOGLYCOSIDE DAMAGE

Aminoglycoside damage in the mammalian hair cells is irreversible and leads to sensorineural hearing loss. Aminoglycoside antibiotics are known to damage the OHCs, but also IHCs and SCs (Murillo-Cuesta et al., 2010; Wu et al., 2001). Aminoglycosides enter HCs through mechanoelectrical transducer channels and inside HCs they form aminoglycoside-iron complexes, which results in the formation of reactive oxygen species (ROS) (Rybak and Ramkuram, 2007). ROS are believed to promote apoptotic cell death. Apoptosis of the HCs begins at the base of the cochlea and spreads up to apical regions (Murillo-Cuesta et al., 2010; Wu et al., 2001). Cheng et al. demonstrated that deletion of the p53 offers a significant protection against cisplatin-induced hair cell loss (Cheng et al., 2005), and Kaur et al. found that siRNA against STAT1 (signal transducer and activator of transcription-1) also offers protection against cisplatin ototoxity (Kaur et al., 2011). Liu et al. concluded in their study that sustained and regulated AAV1-mediated GDNF (Glial cell-derived neurotrophic factor) expression protected the cochlear function of rats from aminoglycoside-induced ototoxicity (Liu et al., 2008). Kanamycin injections caused apoptosis in the cochlear auditory epithelium of CD-1 mice (Figure 9). We observed apoptotic HCs throughout the cochlea. Kanamycin caused apoptosis in both IHCs and OCHs, which was predictable and consistent with our previous results about

lentivirus vectors and also with earlier studies with other mouse strains (Murillo-Cuesta et al., 2010; Pietola et al., 2008; Wu et al., 2001). The degeneration of different inner ear cell types due to aminoglycoside damage was demonstrated by Tunel-staining. We found less apoptotic HCs in the AAV-shRNA injected cochleae than in the contralateral control cochleae and the saline injected control cochleae. The differences in the level of apoptosis between AAV-shRNA (p53 and p27+p53) and saline injected cochleae were statistically significant (Table 7). Surprisingly, the number of apoptotic cells in AAV-p27-shRNA injected mice was not significantly different from that in saline injected mice. These results suggest that silencing of p53 in the kanamycin treated cochleae decreases the cell death in the auditory epithelium.

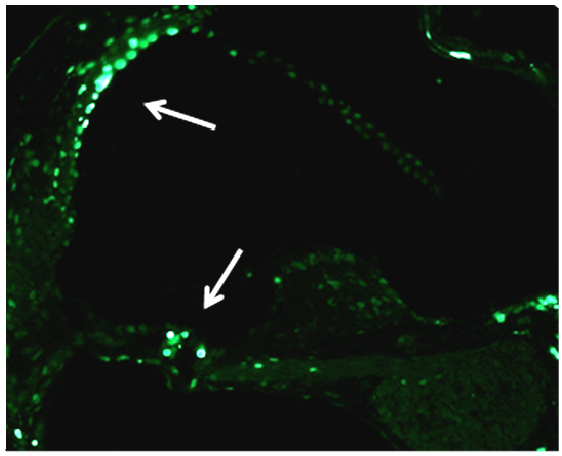


Figure 9. Kanamycin induced apoptosis in the mouse cochlea.

Arrows show apoptotic cells in the cochlea.

Table 7. Level of apoptosis in AAV-shRNA treated cochleae compared to saline control cochleae

Constructs	P value, statistical significance p<0.05
AAV-p53-shRNA vs. saline	p = 0.00014
AAV-p27+p53-shRNA vs. saline	p = 0.0011

5. EFFECT OF AAV-ANTI-CLARIN-1-RIBOZYME IN THE INNER EAR (V)

Ribozymes can be used for a sequence-specific inhibition of gene expression, which makes them potentially useful for gene therapy (Birikh et al., 1997). Self-cleaving ribozymes are small and specific, which enhances their potential for medical applications (Hauswirth et al.,2000). There are two kinds of ribozymes, hairpins and hammerheads, which have both been used in gene therapy studies. Both catalyze sequence-specific cleavage of target RNA, producing molecules which contain a 5' hydroxyl and a 2', 3'-cyclic phosphate. We used hammerhead ribozymes. They have been used more commonly because of their higher specificity to target sites (Birikh et al., 1997; Hauswirth et al., 2000). AAV-delivered ribozymes have not been studied previously in the cochlea, but Hauswirth et al. used vector-delivered ribozymes for the study of retinal diseases (Hauswirth et al., 2000) and Lewin et al. tested ribozymes in rat models of autosomal dominant RP (Lewin et al., 1998).

5.1 CLEAVAGE TIME COURSE REACTIONS

Three hammerhead ribozymes were designed. They all recognized and cleaved wt mouse clarin-1 mRNA. The cleavage time course reactions of three candidate ribozymes were analyzed from a gel autoradiograph in which synthetic RNA oligonucleotide 12-mer substrates were used. We found that the ribozyme Rz166, which cuts right before the start codon following the GUC triplet, was the most efficient *in vitro*, and was consequently chosen for our *in vivo* studies. The ribozyme was attached to an AAV2 vector which had a CBA promoter.

5.2 EXPRESSION OF AAV-GFP IN VIVO

We studied GFP transgene expression in the wt CD-1 mouse cochlea. One week after the injections we saw GFP expression in the hair cells, especially in the IHCs. There was no expression in the SGNs. After one month, GFP fluorescence was visible in both IHCs and OHCs. Again, SGNs did not show any GFP fluorescence. We detected GFP expression in the stria vascularis and in singular epithelial vestibular cells after both one-week and one-month survival. However, the intensity of the GFP expression was variable throughout the cochlea. There was a slight decrease in the expression in the HCs and in the stria vascularis in the one-month samples. Previous studies of the expression of GFP after AAV-GFP-mediated delivery to the cochlea produced similar results (Kilpatrick et al., 2011; Lalwani et al., 1998a,b). Modification of the promoter or the AAV serotype will change the tissue specificity

of the vector and may cause differences in the expression strength (Bedrosian et al., 2006; Kilpatrik et al. 2011; Liu et al., 2005; Luebke et al., 2009). AAV vector using the PDGF promoter allowed transduction of blood vessels in the infused cochlea (Luebke et al., 2001), CAG promoter (a hybrid promoter which contains sequences from CBA and CMV) leads to transduction of exclusively hair cells (Liu et al., 2007; Luebke et al., 2009), myosin VIIA promoter leads to strong and selective transduction of hair cells of cochlea and vestibule (Boeda et al., 2001; Liu et al., 2007), EFS and neuron-specific enolase promoters lead to transduction of spiral ganglion cells and the cells of the spiral ligament (Liu et al., 2007), GFAP promoter has been shown to transduce supporting cells (Rio et al., 2002). The AAV serotype has an effect on the transduction efficiency. Previous studies have reported transduction of HCs in *vivo* with serotypes 1, 2, 3, 5, 7, and 8 (Bedrosian et al., 2006; Liu et al., 2005; Luebke et al., 2009). During our own studies, we have accomplished transduction of HCs *in vivo* with serotypes 1, 2 and 8 (unpublished data; Aarnisalo et al., 2007; Pietola et al., 2012).

5.3 APOPTOSIS AFTER AAV-RIBOZYME TREATMENT

One week after the AAV injection, we detected apoptosis in OHCs, IHCs and in the stria vascularis. We also found few apoptotic cells in vestibular epithelia, whereas SGNs showed no apoptosis. Apoptotic OHCs, IHCs and strial cells were found also one month after the injection. The difference in the number of apoptotic cells between the AAV-Rz and AAV-GFP groups after one-week survival was not statistically significant. The AAV-GFP group showed significantly more apoptotic OHCs and strial cells than the saline control group, and the AAV-Rz group was anyway showing significantly more apoptotic strial cells than the saline control group. We compared the number of apoptotic OHCs, IHCs and strial cells in the AAV-Rz and AAV-GFP groups and found that there was after one-month survival significantly more apoptosis in the AAV-Rz group. Comparison of AAV-GFP to saline control groups showed also significant differences in apoptotic IHCs and strial cells.

This led to the comparison between one-week and one-month results (Table 8). In the AAV-Rz group, significantly more apoptotic OHCs, IHCs, and strial cell were detected in the one-month samples. The vestibular epithelial cells showed no significant difference. In the AAV-GFP group's one-month samples only the number of apoptotic OHCs was significantly increased compared to the one-week samples.

The results suggest that the anti-clarin-1 ribozyme could initiate a process leading to apoptotic cell death in the cochlea. However, it is also possible that the detected apoptosis in the AAV-Rz group is partly an unspecific effect originating from an unspecific breakdown of mRNA. In this case it would not have any relation at all to the apoptosis caused by to clarin-1 loss. The control vector (AAV-GFP) did not

transduce SGNs, thus we did not observe apoptotic SGNs in the AAV-Rz treated group. Since we were not able to deliver the ribozyme cDNA to the spiral ganglion, the effect of anti-clarin-1 ribozymes in SGNs could not be evaluated. The effect of anti-clarin-1 ribozymes on hearing or vestibular functions was not tested and needs further evaluation. The clinical phenotype in USH3A is progressive, suggesting a degenerative process in the cochlea and retina. Our results suggest that an apoptotic process could partly explain the progressive nature of USH3A.

Table 8. Difference in the number of apoptotic cells in the cochlea when comparing one-week and one month samples of AAV-Rz and AAV-GFP groups
(statistical significance $p < 0.05$, marked in grey)

1 wk vs. 1 mo	IHC	OHC	strial cells	vestibular cells
AAV-Rz	$p < 0.0001$	$p < 0.0001$	$p < 0.001$	$p = 0.837$
AAV-GFP	$p = 0.06$	$p = 0.008$	$p = 0.823$	$p = 0.103$

CONCLUDING REMARKS

Usher syndrome type III (USH3) is the most common form of Usher syndrome in Finland. Mutations in Finnish USH3 patients are specific for the Finnish population which makes them an interesting research subject. All our USH3 patients were of Finnish ancestry and they were homozygous for the Finmajor mutation p.Y176X. Even though they all carry the same mutation, their phenotypes vary considerably. To date, only few studies have analyzed the benefits of cochlear implantation in patients with Usher syndrome, and a specific analysis on USH3 patients had not been done earlier. Audiological data, including pure-tone thresholds and speech recognition, were collected and analyzed. The results showed that the patients' postoperative hearing with a cochlear implant was significantly better than their preoperative hearing with a traditional hearing aid. The speech recognition tests showed also significantly better results after cochlear implantation. All patients received a set of questionnaires which evaluated their quality of life. The GBI questionnaire evaluated the patients' health status after otorhinolaryngological intervention, and the GHSI questionnaire contained questions about their health status. The third questionnaire focused on gathering information about the implantation, problems with the use of the cochlear implant, previous use of hearing aids, current status of vision, and vision-related symptoms. The GBI total score was positive, which indicates that USH3 patients believed that their health status was better after the implantation than before it. In conclusion it can be stated that cochlear implantation is beneficial to patients with USH3 and improves their quality of life.

Animal models are widely used in cochlear gene therapy studies. Gene transfer can be achieved via different routes: injection or infusion through the RWM or cochleostomy, or delivery through intact RWM by means of gelatin-sponge soaked with transgene. In order to study less invasive methods of gene transfer through intact RWM, we designed an *in vitro* model of the RWM. First, the permeability of the intact RWM was modified by manipulating its outer surface with $AgNO_3$, trichloroacetic acid or histamine-glycerol. Microscopic inpection of the treated RWMs showed that histamine-glycerol preserved the cellular morphology of the RWM while $AgNO_3$ and trichloroacetic acid destroyed the RWM partially or totally. Secondly, we wanted to test the permeability of the damaged RWM to an adenovirus vector. Toluidine blue was used as a control agent. We found that toluidine blue passed through the treated RWM in a time-dependent manner. Interestingly, damage to the RWM reduced by an unknown mechanism the passage of toluidine blue. The same experiment was repeated with an adenovirus (Ad) vector carrying GFP. Ad-GFP also passed through the intact RWM in a time-depended manner. The passage, however, was slower than that of toluidine blue. We concluded that

the RWM model can be used in gene transfer studies *in vitro*. Treatment of the RWM with $AgNO_3$, trichloroacetic acid or histamine-glycerol damaged the surface of the RWM but did not enhance the passage through the membrane.

Lentivirus vectors have been used in many types on gene transfer studies. Lentiviruses have the ability to infect dividing and non-dividing cells and they insert their genome into the host cells' genome, which creates long and stable expression. Two lentivirus vectors carrying GFP transgene were studied *in vivo*. The vectors were introduced into the inner ear by direct injection through the RWM. Both vectors transduced cells in the scala vestibuli and the scala tympani, but not in the scala media. On the basis of this result it can be concluded that lentivirus vectors can be used in the delivery of transgenes or therapeutic agents to the perilymphatic space. The safety of lentivirus vectors was evaluated as well. Several peripheral organs were analyzed for GFP expression. Only the liver of one mouse contained GFP positive cells. It was concluded that distant spread via the circulatory system is minimal. An analysis of possible immunological reactions to the vectors showed that lentivirus vectors caused only minimal immune responses in the cochlea. The risk of disrupting the immune system within the cochlea by lentivirus delivery seems accordingly low.

Specific genes can be efficiently silenced with RNA interference (RNAi), which can be performed by introducing vector-based short hairpin RNAs (shRNAs) into living cells. The hair cells in the organ of Corti are terminally differentiated. The post-mitotic state is maintained by cyclin-dependent kinase inhibitor $p27^{kip1}$, which acts as a negative regulator in the cell cycle. p53 is a tumor suppressor protein which has a role in DNA damage-induced cell death, and it also initiates cell death in cochlear and vestibular hair cells. Three adeno-associated virus (AAV) vector-delivered shRNAs were created and their effects on the damaged cochlea studied. We found that AAV-p53-shRNA and AAV-p27+p53-shRNA significantly reduced the number of apoptotic cells in the cochleae of mice after kanamycin damage as compared to control animals. The results suggest that shRNAs are suitable for gene silencing experiments in the cochlea, and that silencing of p53 seems to decrease cell death in the kanamycin-damaged cochlea.

Usher syndrome type III is caused by mutations in the *USH3A* gene which encodes clarin 1 (CLRN1) protein. CLRN1 is expressed in the hair cells and spiral ganglion cells, but the function of the protein in the ear is not known. We wanted to examine the effects of AAV-delivered anti-clarin ribozyme (AAV-Rz) in the organ of Corti *in vivo*. The transduction effiency of the used AAV vector was determined by means of the GFP transgene. GFP expression was detected in the hair cells, in the cells of the stria vascularis and in vestibular cells one week and also one month after virus injection, but not at all in spiral ganglion cells. AAV-Rz injections led to apoptosis in the cochlea. After one week few apoptotic hair cells and strial cells were detected. After one month the apoptotic inner and outer hair cells and strial cells

had become significantly more. The number of apoptotic hair cells and strial cells in the AAV-Rz group was one month after the injection also significantly higher than in the AAV-GFP group. There was also a significant difference in the number of apoptotic outer hair cells, inner hair cells, and strial cells in the AAV-Rz group after a week and after a month. These results suggest that AAV-anti-clarin1-ribozyme may cause apoptosis in the sensory epithelia. We also suggest that the apoptotic process could partly explain the progressive nature of USH3.

ACKNOWLEDGEMENTS

Tämä väitöskirja on tehty Helsingin yliopiston lääketieteellisen tiedekunnan korva-, nenä- ja kurkkutautien klinikalla vuosien 2007–2012 aikana. Olen kiitollinen professori Anne Pitkärannalle, entisille professoreille Pekka Karmalle ja Jukka Ylikoskelle sekä dosentti Heikki Rihkaselle ja dosentti Hans Ramsaylle mahdollisuudesta tehdä tutkimustyötä korvaklinikan tutkimustiloissa. Kiitos professori Anne Pitkärannalle suostumisesta väitöstilaisuuden kustokseksi.

I would like to thank Docent Jorma Haapaniemi and Docent Jing Zou, the reviewers of my thesis, for the thorough examination of the thesis and for their valuable and constructive comments on the manuscript.

Ohjaajani Jussi Jerolle, kiitos yhteisistä vuosista tutkimuksen parissa. Uskomatonta ajatella, että yhteistyömme alkoi kuin vahingossa kymmenen vuotta sitten. Loputon optimismisi ja ”luova hulluus” tutkimuksen suhteen on ihailtavaa. Kiitos kaikesta mitä olet minulle opettanut, niin tieteen kuin elämänkin suhteen. Muistan lämmöllä erityisesti kaikkia yhteisiä, hetkittäin hulvattomia, kokousmatkojamme. Tulet aina olemaan minulle tieteen oppi-isä.

Ohjaajani Antti Aarnisalo, en olisi tutkijana se mikä olen ilman sinua. Kiitos että olet tutustuttanut minut tutkimustyöskentelyn saloihin, opettanut lähes kädestä pitäen kirjoittamaan tieteellisiä tekstejä ja suhtautunut aina innostuneesti tutkimukseemme. Työskentely kanssasi on ollut opettavaista ja työt hoituneet aina kuin lennossa.

Syvimmät kiitokseni tutkimusryhmämme jäsenille korvaklinikalla ja Usher-ryhmässä. Usher-ryhmän äiti Eeva-Marja ”Eevis” Sankila, kiitos yhteistyöstä ja siitä, että olet jakanut tietämyksesi Usherista ja tutustuttanut minut tähän kiehtovaan tutkimuskohteeseen. Ehdoton kiitollisuuteni Reetta Vainionpäälle: Tukesi, ystävyytesi ja apusi tutkimustyön aikana on ollut korvaamatonta. Yhdessä vietetyt hetket, olivatpa ne laboratoriossa, ”kellarissa” illan hämärtyessä tai Floridassa palmujen alla, ovat tehneet lähtemättömän vaikutuksen. Hanna Västinsalo, kiinnostuksesi tieteeseen on ihailtavaa. Tukesi ja ystävyytesi etenkin työn loppuvaiheessa on ollut erittäin arvokasta. Teemu Kinnari, iso kiitos avustasi statistiikan kanssa. Työpäiviäni ovat piristäneet myös keskustelumme tieteestä ja kaikesta muusta maan ja taivaan välillä. Haluan myös kiittää Juha Isosomppia avusta Usher tutkimuksen toteutuksessa, hyvästä yhteistyöstä ja lukuisista lennokkaista keskusteluista, joiden aiheena oli useammin jalkapallo kuin Usher. Lämmin kiitos ”kesätytöllemme” Olga ”Inku” Jerolle sekä Joonas Joensuulle avusta tutkimustyössä.

Erityiskiitos ystävälleni Hanna Viisanen-Kuopilalle yhteisistä vuosista Biomedicumin lasikaton alla. Kiitos kaikista kannustavista sanoista ja mielenkiinnosta tutkimustani kohtaan. On ollut ihanaa jakaa tutkimustyön ilot ja

surut ihmisen kanssa, joka todella ymmärtää ne. Tukesi on ollut korvaamatonta. Lisäksi haluan kiittää Mervi Reunasta: olet toiminut esimerkillisenä esikuvana. Kiitos ystävyydestäsi ja tuesta koko väitöskirjatyön aikana.

Haluan osoittaa kiitokseni kaikille yhteistyökumppaneille. Erityisesti haluan kiittää Marjo Salmista uusien menetelmien opettamisesta, sekä Jarmo Wahlforsia ja Riikka Pellistä virusvektorien toimittamisesta.

Kiitos Meilahden tutkimus- ja kehitysyksikön työntekijöille Karille, Ollille, Veikolle ja Virpille avusta eläintöiden kanssa. Anne Reijula, kiitos että olet jakanut ammattiosaamisesi näytteiden käsittelyn suhteen.

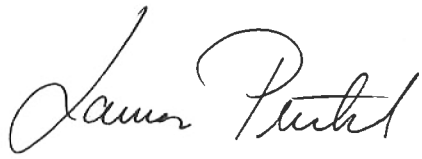
Ihanat ystäväni Anne, Katja ja Toni, Maiju, Tea, Tiina, Samuli ja Reetta, kiitos tuestanne väitöskirjatyön aikana. Mahtavaa, että olette jaksaneet kiinnostua työstäni ja kuunnella selostuksia aiheista, jotka hetkittäin ylittävät ymmärryksenne rajat. Lisäksi haluan kiittää ”bilsatyttöjä” yhteisistä vuosista Viikissä ja Meilahdessa.

Äiti ja isä, sanat eivät riitä kuvaamaan kiitollisuutta jota tunnen. Apunne, onpa se sitten ollut koiranhoitoapua, taloudellista tukea tai kannustusta niinä hetkinä kun oma usko on ollut koetuksella, on ollut täysin korvaamatonta. Tukenne on vaikuttanut oleellisesti tämän väitöskirja valmistumiseen. Erityiskiitokset ATK-tukitiimilleni veljelleni Pasille ja serkulleni Laurille, sekä serkulleni Nooralle upeista kuvista.

Viimeisenä vaan ei vähäisimpänä, valtavan iso kiitos rakkaalle aviomiehelleni Rofalle tuestasi ja etenkin kärsivällisyydestäsi tämän väitöskirjatyön aikana. Mahtavaa, että kotona on ihminen, joka osaa sanoa koska on syytä rauhoittua ja rentoutua. Se on ohi nyt, yritän palata pre-väitöskirja modeen!

This study was funded by the Biomedicum Helsinki Foundation, the Finnish Medical Foundation, the Leo, Mary and Mary-Ann Hackmann Foundation, the Maud Kuistila Memorial Foundation, the Medicinska Understödsföreningen Liv och Hälsa, the Sigrid Jusélius foundation, the Oscar Öflund Foundation, the University of Helsinki, and the Helsinki University Central Hospital Reseach Funds.

Helsingissä 7.5.2012



REFERENCES

Aarnisalo AA, Aarnisalo P, Pietola L, Wahlfors J, Jero J (2006) Efficacy of gene transfer through the round window membrane: an in vitro model. ORL J Otorhinolaryngol Relat Spec 68:220-227.

Aarnisalo AA, Pietola L, Joensuu J, Isosomppi J, Aarnisalo P, Dinculescu A, Lewin AS, Flannery J, Hauswirth WW, Sankila EM, Jero J (2007) Anti-clarin-1 AAV-delivered ribozyme induced apoptosis in the mouse cochlea. Hear Res 230:9-16.

Adato A, Vreugde S, Joensuu T, Avidan N, Hamalainen R, Belenkiy O, Olender T, Bonne-Tamir B, Ben-Asher E, Espinos C, Millan JM, Lehesjoki AE, Flannery JG, Avraham KB, Pietrokovski S, Sankila EM, Beckmann JS,Lancet D (2002) USH3A transcripts encode clarin-1, a four-transmembrane-domain protein with a possible role in sensory synapses. Eur J Hum Genet 10:339-350.

Adato A, Michel V, Kikkawa Y, Reiners J, Alagramam KN, Weil D, Yonekawa H, Wolfrum U, El-Amraoui A, Petit C (2005) Interactions in the network of Usher syndrome type 1 proteins. Hum Mol Genet 14:347-356.

Admiraal RJ, Szymko YM, Griffith AJ, Brunner HG, Huygen PL (2002) Hearing impairment in Stickler syndrome. Adv Otorhinolaryngol 61:216-23.

Ahmed ZM, Riazuddin S, Bernstein SL, Ahmed Z, Khan S, Griffith AJ, Morell RJ, Friedman TB, Riazuddin S,Wilcox ER (2001) Mutations of the protocadherin gene PCDH15 cause Usher syndrome type 1F. Am J Hum Genet 69:25-34.

Ahmed ZM, Riazuddin S, Khan SN, Friedman PL, Riazuddin S,Friedman TB (2009) USH1H, a novel locus for type I Usher syndrome, maps to chromosome 15q22-23. Clin Genet 75:86-91.

Alagramam KN, Yuan H, Kuehn MH, Murcia CL, Wayne S, Srisailpathy CR, Lowry RB, Knaus R, Van Laer L, Bernier FP, Schwartz S, Lee C, Morton CC, Mullins RF, Ramesh A, Van Camp G, Hageman GS, Woychik RP,Smith RJ (2001) Mutations in the novel protocadherin PCDH15 cause Usher syndrome type 1F. Hum Mol Genet 10:1709-1718.

Aller E, Jaijo T, Oltra S, Alio J, Galan F, Najera C, Beneyto M,Millan JM (2004) Mutation screening of USH3 gene (clarin-1) in Spanish patients with Usher syndrome: low prevalence and phenotypic variability. Clin Genet 66:525-529.

Arnberg N (2009) Adenovirus receptors: implications for tropism, treatment and targeting. Rev Med Virol 19(3):165-78.

Bedrosian JC, Gratton MA, Brigande JV, Tang W, Landau J,Bennett J (2006) In vivo delivery of recombinant viruses to the fetal murine cochlea: transduction characteristics and long-term effects on auditory function. Mol Ther 14:328-335.

Bergelson JM (1999) Receptors mediating adenovirus attachment and internalization. Biochem Pharmacol 57:975-979.

Berger W (1998) Molecular dissection of Norrie disease. Acta Anat (Basel) 162(2-3):95-100.

Berne RM, Levy MN (2003) Physiology. St. Louis: Mosby Book, cop.

Bessis N, GarciaCozar FJ, Boissier MC (2004) Immune responses to gene therapy vectors: influence on vector function and effector mechanisms. Gene Ther 11 Suppl 1:S10-7.

Birikh KR, Berlin YA, Soreq H, Eckstein F (1997) Probing accessible sites for ribozymes on human acetylcholinesterase RNA. RNA 3:429-437.

Bitner-Glindzicz M, Tranebjaerg L (2000) The Jervell and Lange-Nielsen syndrome. Adv Otorhinolaryngol 56:45-52.

Boeda B, Weil D, Petit C (2001) A specific promoter of the sensory cells of the inner ear defined by transgenesis. Hum Mol Genet 10:1581-1589.

Bolz H, von Brederlow B, Ramirez A, Bryda EC, Kutsche K, Nothwang HG, Seeliger M, del C-Salcedo Cabrera M, Vila MC, Molina OP, Gal A, Kubisch C (2001) Mutation of CDH23, encoding a new member of the cadherin gene family, causes Usher syndrome type 1D. Nat Genet 27:108-112.

Boughman JA, Vernon M, Shaver KA (1983) Usher syndrome: definition and estimate of prevalence from two high-risk populations. J Chronic Dis 36:595-603.

Carvalho GJ, Lalwani AK (1999) The effect of cochleostomy and intracochlear infusion on auditory brain stem response threshold in the guinea pig. Am J Otol 20:87-90.

Casto BC, Atchison RW, Hammon WM (1967) Studies on the relationship between adeno-associated virus type I (AAV-1) and adenoviruses. I. Replication of AAV-1 in certain cell cultures and its effect on helper adenovirus. Virology 32:52-59.

Chaib H, Kaplan J, Gerber S, Vincent C, Ayadi H, Slim R, Munnich A, Weissenbach J,Petit C (1997) A newly identified locus for Usher syndrome type I, USH1E, maps to chromosome 21q21. Hum Mol Genet 6:27-31.

Chen G, Zhang X, Yang F, Mu L (2010) Disposition of nanoparticle-based delivery system via inner ear administration. Curr Drug Metab 11:886-897.

Chen P, Segil N (1999) p27(Kip1) links cell proliferation to morphogenesis in the developing organ of Corti. Development 126:1581-1590.

Chen P, Zindy F, Abdala C, Liu F, Li X, Roussel MF, Segil N (2003) Progressive hearing loss in mice lacking the cyclin-dependent kinase inhibitor Ink4d. Nat Cell Biol 5:422-426.

Cheng AG, Cunningham LL, Rubel EW (2005) Mechanisms of hair cell death and protection. Curr Opin Otolaryngol Head Neck Surg 13:343-348.

Clements JE, Narayan O (1981) A physical map of the linear unintegrated DNA of Visna virus. Virology 113:412-415.

Cohen CJ, Shieh JT, Pickles RJ, Okegawa T, Hsieh JT, Bergelson JM (2001) The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction Proc Natl Acad Sci U S A 18;98(26):15191-6.

Coleman B, de Silva MG, Shepherd RK (2007) Concise review: the potential of stem cells for auditory neuron generation and replacement. Stem Cells 25:2685-2694.

Collado MS, Burns JC, Hu Z,Corwin JT (2008) Recent advances in hair cell regeneration research. Curr Opin Otolaryngol Head Neck Surg 16:465-471.

Corwin JT, Cotanche DA (1988) Regeneration of sensory hair cells after acoustic trauma. Science 240:1772-1774.

David EE, Ostroff JM, Shipp D, Nedzelski JM, Chen JM, Parnes LS, Zimmerman K, Schramm D, Seguin C (2003) Speech coding strategies and revised cochlear implant candidacy: an analysis of post-implant performance. Otol Neurotol 24:228-233.

Dechecchi MC, Melotti P, Bonizzato A, Santacatterina M, Chilosi M, Cabrini G (2001) Heparan sulfate glycosaminoglycans are receptors sufficient to mediate the initial binding of adenovirus types 2 and 5. J Virol 75:8772-8780.

Dennis RG, Whitmire RN, Jackson RT (1976) Action of inflammatory mediators on middle ear mucosa. A method for measuring permeability and swelling. Arch Otolaryngol 102(7):420-4.

Dror AA, Avraham KB (2009) Hearing loss: mechanisms revealed by genetics and cell biology. Annu Rev Genet 43:411-437.

Ebermann I, Scholl HP, Charbel Issa P, Becirovic E, Lamprecht J, Jurklies B, Millan JM, Aller E, Mitter D, Bolz H (2007) A novel gene for Usher syndrome type 2: mutations in the long isoform of whirlin are associated with retinitis pigmentosa and sensorineural hearing loss. Hum Genet 121:203-211.

Ebermann I, Phillips JB, Liebau MC, Koenekoop RK, Schermer B, Lopez I, Schäfer E, Roux AF, Dafinger C, Bernd A, Zrenner E, Claustres M, Blanco B, Nürnberg G, Nürnberg P, Ruland R, Westerfield M, Benzing T, Bolz HJ (2010) PDZD7 is a modifier of retinal disease and a contributor to digenic Usher syndrome. J Clin Invest 120(6):1812-23.

Eudy JD, Yao S, Weston MD, Ma-Edmonds M, Talmadge CB, Cheng JJ, Kimberling WJ, Sumegi J (1998) Isolation of a gene encoding a novel member of the nuclear receptor superfamily from the critical region of Usher syndrome type IIa at 1q41. Genomics 50:382-384.

Fekete DM, Rouiller EM, Liberman MC, Ryugo DK (1984) The central projections of intracellularly labeled auditory nerve fibers in cats. J Comp Neurol 229:432-450.

Ferrari FK, Samulski T, Shenk T, Samulski RJ (1996) Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. J Virol 70(5):3227-34.

Fields RR, Zhou G, Huang D, Davis JR, Moller C, Jacobson SG, Kimberling WJ, Sumegi J (2002) Usher syndrome type III: revised genomic structure of the USH3 gene and identification of novel mutations. Am J Hum Genet 71:607-617.

Flynn AN, Itani OA, Moninger TO, Welsh MJ (2009) Acute regulation of tight junction ion selectivity in human airway epithelia. Proc Natl Acad Sci U S A 106(9):3591-6.

Friedman TB, Griffith AJ (2003) Human nonsyndromic sensorineural deafness. Annu Rev Genomics Hum Genet 4:341-402.

Gantz BJ, Woodworth GG, Knutson JF, Abbas PJ,Tyler RS (1993) Multivariate predictors of audiological success with multichannel cochlear implants. Ann Otol Rhinol Laryngol 102:909-916.

Geng R, Geller SF, Hayashi T, Ray CA, Reh TA, Bermingham-McDonogh O, Jones SM, Wright CG, Melki S, Imanishi Y, Palczewski K, Alagramam KN, Flannery JG (2009) Usher syndrome IIIA gene clarin-1 is essential for hair cell function and associated neural activation. Hum Mol Genet 18:2748-2760.

Gerber S, Bonneau D, Gilbert B, Munnich A, Dufier JL, Rozet JM, Kaplan J (2006) USH1A: chronicle of a slow death. Am J Hum Genet 78(2):357-9.

Glaser B (2003) Pendred syndrome. Pediatr Endocrinol Rev 1 Suppl 2:199-204.

Goncalves MA (2005) Adeno-associated virus: from defective virus to effective vector. Virol J 2:43.

Gorlin RJ, Toriello HV, Cohen MM, (1995) Hereditary hearing loss and its syndromes. Oxford: Oxford University Press.

Goycoolea MV, Muchow D, Schachern P (1988) Experimental studies on round window structure: function and permeability. Laryngoscope 98:1-20.

Goycoolea MV (1992) The round window membrane under normal and pathological conditions. Acta Otolaryngol Suppl 493:43-55.

Goycoolea MV (1995) Oval and round window membrane changes in otitis media in the human. An ultrastructural study. Acta Otolaryngol 115:282-285.

Greber UF, Willetts M, Webster P, Helenius A (1993) Stepwise dismantling of adenovirus 2 during entry into cells. Cell 75:477-486.

Guilford P, Ben Arab S, Blanchard S, Levilliers J, Weissenbach J, Belkahia A, Petit C (1994) A non-syndrome form of neurosensory, recessive deafness maps to the pericentromeric region of chromosome 13q. Nat Genet 6:24-28.

Guntaka RV (1993) Transcription termination and polyadenylation in retroviruses. Microbiol Rev 57:511-521.

Gurtler N, Lalwani AK (2002) Etiology of syndromic and nonsyndromic sensorineural hearing loss. Otolaryngol Clin North Am 35:891-908.

Han JJ, Mhatre AN, Wareing M, Pettis R, Gao WQ, Zufferey RN, Trono D, Lalwani AK (1999) Transgene expression in the guinea pig cochlea mediated by a lentivirus-derived gene transfer vector. Hum Gene Ther 10:1867-1873.

Harper JW (2001) Protein destruction: adapting roles for Cks proteins. Curr Biol 11:R431-5.

Haseltine WA (1991) Molecular biology of the human immunodeficiency virus type 1. FASEB J 5:2349-2360.

Hauswirth WW, LaVail MM, Flannery JG, Lewin AS (2000) Ribozyme gene therapy for autosomal dominant retinal disease. Clin Chem Lab Med 38:147-153.

Herrera W, Aleman TS, Cideciyan AV, Roman AJ, Banin E, Ben-Yosef T, Gardner LM, Sumaroka A, Windsor EA, Schwartz SB, Stone EM, Liu XZ, Kimberling WJ, Jacobson SG (2008) Retinal disease in Usher syndrome III caused by mutations in the clarin-1 gene. Invest Ophthalmol Vis Sci 49:2651-2660.

Hess JL, Pyper JM, Clements JE (1986) Nucleotide sequence and transcriptional activity of the caprine arthritis-encephalitis virus long terminal repeat. J Virol 60:385-393.

Holt JR (2002) Viral-mediated gene transfer to study the molecular physiology of the Mammalian inner ear. Audiol Neurootol 7(3):157-60.

Horbelt CV (2008) A review of physical, behavioral, and oral characteristics associated with Treacher Collins syndrome, Goldenhar syndrome, and Angelman syndrome. Gen Dent 56(5):416-9.

Hudspeth AJ (1989) How the ear's works work. Nature 341:397-404

Hudspeth AJ (2005) How the ear's works work: mechanoelectrical transduction and amplification by hair cells. C R Biol 328:155-162.

Isosomppi J, Vastinsalo H, Geller SF, Heon E, Flannery JG, Sankila EM (2009) Disease-causing mutations in the CLRN1 gene alter normal CLRN1 protein trafficking to the plasma membrane. Mol Vis 15:1806-1818.

Jahn AF, Santos-Sacchi J (2001) Physiology of the ear. Singular.

Jero J, Mhatre AN, Tseng CJ, Stern RE, Coling DE, Goldstein JA, Hong K, Zheng WW, Hoque AT, Lalwani AK (2001a) Cochlear gene delivery through an intact round window membrane in mouse. Hum Gene Ther 12:539-548.

Jero J, Tseng CJ, Mhatre AN, Lalwani AK (2001b) A surgical approach appropriate for targeted cochlear gene therapy in the mouse. Hear Res 151:106-114.

Joensuu T, Hamalainen R, Yuan B, Johnson C, Tegelberg S, Gasparini P, Zelante L, Pirvola U, Pakarinen L, Lehesjoki AE, de la Chapelle A, Sankila EM (2001) Mutations in a novel gene with transmembrane domains underlie Usher syndrome type 3. Am J Hum Genet 69:673-684.

Juhn SK, Hamaguchi Y, Goycoolea M (1989) Review of round window membrane permeability. Acta Otolaryngol Suppl 457:43-8.

Kandell E, Schwartz J, Jessel T, (2000) Principles of neural science.

Kandler K, Clause A, Noh J (2009) Tonotopic reorganization of developing auditory brainstem circuits. Nat Neurosci 12:711-717.

Kaplan J, Gerber S, Bonneau D, Rozet JM, Delrieu O, Briard ML, Dollfus H, Ghazi I, Dufier JL, Frézal J, et al. (1992) A gene for Usher syndrome type I (USH1A) maps to chromosome 14q. Genomics 14(4):979-87.

Karjalainen S, Pakarinen L, Terasvirta M, Kaariainen H, Vartiainen E (1989) Progressive hearing loss in Usher's syndrome. Ann Otol Rhinol Laryngol 98:863-866.

Kashtan CE (1999) Alport syndrome. An inherited disorder of renal, ocular, and cochlear basement membranes. Medicine (Baltimore) 78(5):338-60.

Kaur T, Mukherjea D, Sheehan K, Jajoo S, Rybak LP, Ramkumar V (2011) Short interfering RNA against STAT1 attenuates cisplatin-induced ototoxicity in the rat by suppressing inflammation. Cell Death Dis 2:e180.

Keats BJ, Berlin CI (1999) Genomics and hearing impairment. Genome Res 9:7-16.

Keats BJ, Savas S (2004) Genetic heterogeneity in Usher syndrome. Am J Med Genet A 130A:13-16.

Kesser BW, Lalwani AK (2009) Gene therapy and stem cell transplantation: strategies for hearing restoration. Adv Otorhinolaryngol 66:64-86.

Kierszenbaum AL (2002) Histology and Cell Biology: An Introduction to Pathology. St. Louis: Mosby.

Kilpatrick LA, Li Q, Yang J, Goddard JC, Fekete DM, Lang H (2011) Adeno-associated virus-mediated gene delivery into the scala media of the normal and deafened adult mouse ear. Gene Ther 18:569-578.

Kochhar A, Fischer SM, Kimberling WJ, Smith RJ (2007) Branchio-oto-renal syndrome. Am J Med Genet A 143A (14):1671-8.

Kokotas H, Petersen MB, Willems PJ (2007) Mitochondrial deafness. Clin Genet 71:379-391.

Kremer H, van Wijk E, Marker T, Wolfrum U, Roepman R (2006) Usher syndrome: molecular links of pathogenesis, proteins and pathways. Hum Mol Genet 15 Spec No 2:R262-70.

Laine H, Doetzlhofer A, Mantela J, Ylikoski J, Laiho M, Roussel MF, Segil N, Pirvola U (2007) p19(Ink4d) and p21(Cip1) collaborate to maintain the postmitotic state of auditory hair cells, their codeletion leading to DNA damage and p53-mediated apoptosis. J Neurosci 27:1434-1444.

Lalwani AK, Walsh BJ, Reilly PG, Muzyczka N, Mhatre AN (1996) Development of in vivo gene therapy for hearing disorders: introduction of adeno-associated virus into the cochlea of the guinea pig. Gene Ther 3(7):588-92.

Lalwani AK, Han JJ, Walsh BJ, Zolotukhin S, Muzyczka N, Mhatre AN (1997) Green fluorescent protein as a reporter for gene transfer studies in the cochlea. Hear Res 114(1-2):139-47.

Lalwani A, Walsh B, Reilly P, Carvalho G, Zolotukhin S, Muzyczka N, Mhatre A (1998a) Long-term in vivo cochlear transgene expression mediated by recombinant adeno-associated virus. Gene Ther 5:277-281.

Lalwani AK, Walsh BJ, Carvalho GJ, Muzyczka N, Mhatre AN (1998b) Expression of adeno-associated virus integrated transgene within the mammalian vestibular organs. Am J Otol 19:390-395.

Lalwani AK, Jero J, Mhatre AN (2002a) Current issues in cochlear gene transfer. Audiol Neurootol 7:146-151.

Lalwani AK, Jero J, Mhatre AN (2002b) Developments in cochlear gene therapy. Adv Otorhinolaryngol 61:28-33.

LeMasurier M, Gillespie PG (2005) Hair-cell mechanotransduction and cochlear amplification. Neuron 48:403-415.

Lewin AS, Drenser KA, Hauswirth WW, Nishikawa S, Yasumura D, Flannery JG, LaVail MM (1998) Ribozyme rescue of photoreceptor cells in a transgenic rat model of autosomal dominant retinitis pigmentosa. Nat Med 4:967-971.

Lim DJ, Kalinec F (1998) Cell and molecular basis of hearing. Kidney Int Suppl 65:S104-13.

Liu Y, Okada T, Sheykholeslami K, Shimazaki K, Nomoto T, Muramatsu S, Kanazawa T, Takeuchi K, Ajalli R, Mizukami H, Kume A, Ichimura K, Ozawa K (2005) Specific and efficient transduction of Cochlear inner hair cells with recombinant adeno-associated virus type 3 vector. Mol Ther 12:725-733.

Liu Y, Okada T, Nomoto T, Ke X, Kume A, Ozawa K, Xiao S (2007) Promoter effects of adeno-associated viral vector for transgene expression in the cochlea in vivo. Exp Mol Med 39:170-175.

Liu Y, Okada T, Shimazaki K, Sheykholeslami K, Nomoto T, Muramatsu S, Mizukami H, Kume A, Xiao S, Ichimura K, Ozawa K (2008) Protection against aminoglycoside-induced ototoxicity by regulated AAV vector-mediated GDNF gene transfer into the cochlea. Mol Ther 16:474-480.

Loundon N, Marlin S, Busquet D, Denoyelle F, Roger G, Renaud F, Garabedian EN (2003) Usher syndrome and cochlear implantation. Otol Neurotol 24:216-221.

Lowenheim H, Furness DN, Kil J, Zinn C, Gultig K, Fero ML, Frost D, Gummer AW, Roberts JM, Rubel EW, Hackney CM, Zenner HP (1999) Gene disruption of p27(Kip1) allows cell proliferation in the postnatal and adult organ of corti. Proc Natl Acad Sci U S A 96:4084-4088.

Luebke AE, Foster PK, Muller CD, Peel AL (2001a) Cochlear function and transgene expression in the guinea pig cochlea, using adenovirus- and adeno-associated virus-directed gene transfer. Hum Gene Ther 12:773-781.

Luebke AE, Steiger JD, Hodges BL, Amalfitano A (2001b) A modified adenovirus can transfect cochlear hair cells in vivo without compromising cochlear function. Gene Ther 8:789-794.

Luebke AE, Rova C, Von Doersten PG, Poulsen DJ (2009) Adenoviral and AAV-mediated gene transfer to the inner ear: role of serotype, promoter, and viral load on in vivo and in vitro infection efficiencies. Adv Otorhinolaryngol 66:87-98.

Maerker T, van Wijk E, Overlack N, Kersten FF, McGee J, Goldmann T, Sehn E, Roepman R, Walsh EJ, Kremer H, Wolfrum U (2008) A novel Usher protein network at the periciliary reloading point between molecular transport machineries in vertebrate photoreceptor cells. Hum Mol Genet 17:71-86.

Manchaiah VK, Zhao F, Danesh AA, Duprey R (2011) The genetic basis of auditory neuropathy spectrum disorder (ANSD). Int J Pediatr Otorhinolaryngol 75(2):151-8.

Mantela J, Jiang Z, Ylikoski J, Fritzsch B, Zacksenhaus E, Pirvola U (2005) The retinoblastoma gene pathway regulates the postmitotic state of hair cells of the mouse inner ear. Development 132:2377-2388.

Mason JC, De Michele A, Stevens C, Ruth RA, Hashisaki GT (2003) Cochlear implantation in patients with auditory neuropathy of varied etiologies. Laryngoscope 113(1):45-9.

Meyer AC, Frank T, Khimich D, Hoch G, Riedel D, Chapochnikov NM, Yarin YM, Harke B, Hell SW, Egner A, Moser T (2009) Tuning of synapse number, structure and function in the cochlea. Nat Neurosci 12:444-453.

Miriszlai E, Benedeczky I, Csapo S, Bodanszky H (1978) The ultrastructure of the round window membrane of the cat. ORL J Otorhinolaryngol Relat Spec 40:111-119.

Morsli H, Choo D, Ryan A, Johnson R, Wu DK (1998) Development of the mouse inner ear and origin of its sensory organs. J Neurosci 18:3327-3335.

Morton CC, Nance WE (2006) Newborn hearing screening--a silent revolution. N Engl J Med 354:2151-2164.

Mukherjea D, Jajoo S, Sheehan K, Kaur T, Sheth S, Bunch J, Perro C, Rybak LP, Ramkumar V (2011) NOX3 NADPH oxidase couples transient receptor potential vanilloid 1 to signal transducer and activator of transcription 1-mediated inflammation and hearing loss. Antioxid Redox Signal 14:999-1010.

Murillo-Cuesta S, Contreras J, Cediel R, Varela-Nieto I (2010) Comparison of different aminoglycoside antibiotic treatments to refine ototoxicity studies in adult mice. Lab Anim 44:124-131.

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272:263-267.

Ness SL, Ben-Yosef T, Bar-Lev A, Madeo AC, Brewer CC, Avraham KB, Kornreich R, Desnick RJ, Willner JP, Friedman TB, Griffith AJ (2003) Genetic homogeneity and phenotypic variability among Ashkenazi Jews with Usher syndrome type III. J Med Genet 40:767-772.

Nguyen DH, Hildreth JE (2000) Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. J Virol 74:3264-3272.

Ni TH, McDonald WF, Zolotukhin I, Melendy T, Waga S, Stillman B, Muzyczka N (1998) Cellular proteins required for adeno-associated virus DNA replication in the absence of adenovirus coinfection. J Virol 72:2777-2787.

Oshima K, Shin K, Diensthuber M, Peng AW, Ricci AJ, Heller S (2010) Mechanosensitive hair cell-like cells from embryonic and induced pluripotent stem cells. Cell 141:704-716.

Ottersen OP, Takumi Y, Matsubara A, Landsend AS, Laake JH, Usami S (1998) Molecular organization of a type of peripheral glutamate synapse: the afferent synapses of hair cells in the inner ear. Prog Neurobiol 54:127-148.

Pakarinen L, Tuppurainen K, Laippala P, Mantyjarvi M, Puhakka H (1995) The ophthalmological course of Usher syndrome type III. Int Ophthalmol 19:307-311.

Paterna JC, Moccetti T, Mura A, Feldon J, Bueler H (2000) Influence of promoter and WHV post-transcriptional regulatory element on AAV-mediated transgene expression in the rat brain. Gene Ther 7:1304-1311.

Pellinen R, Hakkarainen T, Wahlfors T, Tulimäki K, Ketola A, Tenhunen A, Salonen T, Wahlfors J (2004) Cancer cells as targets for lentivirus-mediated gene transfer and gene therapy. Int J Oncol 25(6):1753-62.

Petersen MB (2002) Non-syndromic autosomal-dominant deafness. Clin Genet 62:1-13.

Petersen MB, Willems PJ (2006) Non-syndromic, autosomal-recessive deafness. Clin Genet 69:371-392.

Petersen MB, Wang Q, Willems PJ (2008) Sex-linked deafness. Clin Genet 73:14-23.

Pietola L, Aarnisalo AA, Joensuu J, Pellinen R, Wahlfors J, Jero J (2008) HOX-GFP and WOX-GFP lentivirus vectors for inner ear gene transfer. Acta Otolaryngol 128:613-620.

Pietola L, Jero J, Jalkanen R, Kinnari TJ, Jero O, Frilander M, Pajusola K, Salminen M, Aarnisalo A (2012) Effects of p27kip1- and p53-shRNAs on kanamycin damaged mouse cochlea. World J Otorhinolaryngol 2(1):1-7.

Praetorius M, Knipper M, Schick B, Tan J, Limberger A, Carnicero E, Alonso MT, Schimmang T (2002) A novel vestibular approach for gene transfer into the inner ear. Audiol Neurootol 7:324-334.

Purcell DF, Martin MA (1993) Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication, and infectivity. J Virol 67:6365-6378.

Pyper JM, Clements JE, Gonda MA, Narayan O (1986) Sequence homology between cloned caprine arthritis encephalitis virus and visna virus, two neurotropic lentiviruses. J Virol 58:665-670.

Raphael Y, Frisancho JC, Roessler BJ (1996) Adenoviral-mediated gene transfer into guinea pig cochlear cells in vivo. Neurosci Lett 207:137-141.

Raphael Y, Altschuler RA (2003) Structure and innervation of the cochlea. Brain Res Bull 60:397-422.

Ratner L, Haseltine W, Patarca R, Livak KJ, Starcich B, Josephs SF, Doran ER, Rafalski JA, Whitehorn EA, Baumeister K (1985) Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature 313:277-284.

Read AP (2000) Waardenburg syndrome. Adv Otorhinolaryngol 56:32-8.

Reiners J, van Wijk E, Marker T, Zimmermann U, Jurgens K, te Brinke H, Overlack N, Roepman R, Knipper M, Kremer H, Wolfrum U (2005) Scaffold protein harmonin (USH1C) provides molecular links between Usher syndrome type 1 and type 2. Hum Mol Genet 14:3933-3943.

Reiners J, Nagel-Wolfrum K, Jurgens K, Marker T, Wolfrum U (2006) Molecular basis of human Usher syndrome: deciphering the meshes of the Usher protein network provides insights into the pathomechanisms of the Usher disease. Exp Eye Res 83:97-119.

Richardson TL, Ishiyama E, Keels EW (1971) Submicroscopic studies of the round window membrane. Acta Otolaryngol 71:9-21.

Rio C, Dikkes P, Liberman MC, Corfas G (2002) Glial fibrillary acidic protein expression and promoter activity in the inner ear of developing and adult mice. J Comp Neurol 442:156-162.

Ryals BM, Rubel EW (1988) Hair cell regeneration after acoustic trauma in adult Coturnix quail. Science 240:1774-1776.

Rybak LP, Ramkumar V (2007) Ototoxicity. Kidney Int 72:931-935.

Ryugo DK (2008) Projections of low spontaneous rate, high threshold auditory nerve fibers to the small cell cap of the cochlear nucleus in cats. Neuroscience 154:114-126.

Sadeghi M, Cohn ES, Kimberling WJ, Tranebjaerg L, Moller C (2005) Audiological and vestibular features in affected subjects with USH3: a genotype/phenotype correlation. Int J Audiol 44:307-316.

Sage C, Huang M, Karimi K, Gutierrez G, Vollrath MA, Zhang DS, Garcia-Anoveros J, Hinds PW, Corwin JT, Corey DP, Chen ZY (2005) Proliferation of functional hair cells in vivo in the absence of the retinoblastoma protein. Science 307:1114-1118.

Saihan Z, Webster AR, Luxon L, Bitner-Glindzicz M (2009) Update on Usher syndrome. Curr Opin Neurol 22:19-27.

Salmon P, Kindler V, Ducrey O, Chapuis B, Zubler RH, Trono D (2000) High-level transgene expression in human hematopoietic progenitors and differentiated blood lineages after transduction with improved lentiviral vectors. Blood 96(10):3392-8.

Sankila EM, Pakarinen L, Kaariainen H, Aittomaki K, Karjalainen S, Sistonen P, de la Chapelle A (1995) Assignment of an Usher syndrome type III (USH3) gene to chromosome 3q. Hum Mol Genet 4:93-98.

SanlavilleD, Verlos A (2007) CHARGE syndrome: an update. Eur J Hum Genet 15(4):389-99.

Schrijver I, Gardner P (2006) Hereditary sensorineural hearing loss: advances in molecular genetics and mutation analysis. Expert Rev Mol Diagn 6:375-386.

Shibata SB, Raphael Y (2010) Future approaches for inner ear protection and repair. J Commun Disord 43:295-310.

Sinnathuray AR, Raut V, Awa A, Magee A, Toner JG (2003) A review of cochlear implantation in mitochondrial sensorineural hearing loss. Otol Neurotol 24:418-426.

Staecker H, Li D, O'Malley BW,Jr, Van De Water TR (2001) Gene expression in the mammalian cochlea: a study of multiple vector systems. Acta Otolaryngol 121:157-163.

Summerford C, Samulski RJ (1998) Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J Virol 72:1438-1445.

Tian G, Zhou Y, Hajkova D, Miyagi M, Dinculescu A, Hauswirth WW, Palczewski K, Geng R, Alagramam KN, Isosomppi J, Sankila EM, Flannery JG, Imanishi Y (2009) Clarin-1, encoded by the Usher Syndrome III causative gene, forms a membranous microdomain: possible role of clarin-1 in organizing the actin cytoskeleton. J Biol Chem 284:18980-18993.

Teagle HF, Roush PA, Woodard JS, Hatch DR, Zdanski CJ, Buss E, Buchman CA (2010) Cochlear implantation in children with auditory neuropathy spectrum disorder. Ear Hear 31(3):325-35.

UK Cochlear Implant Study Group (2004) Criteria of candidacy for unilateral cochlear implantation in postlingually deafened adults I: theory and measures of effectiveness. Ear Hear 25(4):310-35.

van Dijk JE, van Olphen AF, Langereis MC, Mens LH, Brokx JP, Smoorenburg GF (1999) Predictors of cochlear implant performance. Audiology 38:109-116.

van Wijk E, Pennings RJ, te Brinke H, Claassen A, Yntema HG, Hoefsloot LH, Cremers FP, Cremers CW, Kremer H (2004) Identification of 51 novel exons of the Usher syndrome type 2A (USH2A) gene that encode multiple conserved functional domains and that are mutated in patients with Usher syndrome type II. Am J Hum Genet 74:738-744.

Varmus H (1988) Retroviruses. Science 240:1427-1435.

Verpy E, Leibovici M, Zwaenepoel I, Liu XZ, Gal A, Salem N, Mansour A, Blanchard S, Kobayashi I, Keats BJ, Slim R, Petit C (2000) A defect in harmonin, a PDZ domain-containing protein expressed in the inner ear sensory hair cells, underlies Usher syndrome type 1C. Nat Genet 26:51-55.

Västinsalo H, Jalkanen R, Dinculescu A, Isosomppi J, Geller S, Flannery JG, Hauswirth WW, Sankila EM (2011) Alternative splice variants of the USH3A gene Clarin 1 (CLRN1). Eur J Hum Genet 19(1):30-5.

Walters RW, Yi SM, Keshavjee S, Brown KE, Welsh MJ, Chiorini JA, Zabner J (2001) Binding of adeno-associated virus type 5 to 2,3-linked sialic acid is required for gene transfer. J Biol Chem 276(23):20610-6.

Walters RW, Freimuth P, Moninger TO, Ganske I, Zabner J, Welsh MJ (2002) Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. Cell 20;110(6):789-99.

Waltzman SB, Cohen NL, Shapiro WH (1992) Use of a multichannel cochlear implant in the congenitally and prelingually deaf population. Laryngoscope 102:395-399.

Wang H, Murphy R, Taaffe D, Yin S, Xia L, Hauswirth WW, Bance M, Robertson GS, Wang J (2011) Efficient cochlear gene transfection in guinea-pigs with adeno-associated viral vectors by partial digestion of round window membrane. Gene Ther 19(3):255-63.

Wang QJ, Lu CY, Li N, Rao SQ, Shi YB, Han DY, Li X, Cao JY, Yu LM, Li QZ, Guan MX, Yang WY, Shen Y (2004) Y-linked inheritance of non-syndromic hearing impairment in a large Chinese family. J Med Genet 41:e80.

Wareing M, Mhatre AN, Pettis R, Han JJ, Haut T, Pfister MH, Hong K, Zheng WW, Lalwani AK (1999) Cationic liposome mediated transgene expression in the guinea pig cochlea. Hear Res 128:61-69.

Wayne S, Der Kaloustian VM, Schloss M, Polomeno R, Scott DA, Hejtmancik JF, Sheffield VC, Smith RJ (1996) Localization of the Usher syndrome type ID gene (Ush1D) to chromosome 10. Hum Mol Genet 5:1689-1692.

Weil D, Blanchard S, Kaplan J, Guilford P, Gibson F, Walsh J, Mburu P, Varela A, Levilliers J, Weston MD (1995) Defective myosin VIIA gene responsible for Usher syndrome type 1B. Nature 374:60-61.

Weil D, El-Amraoui A, Masmoudi S, Mustapha M, Kikkawa Y, Laine S, Delmaghani S, Adato A, Nadifi S, Zina ZB, Hamel C, Gal A, Ayadi H, Yonekawa H, Petit C (2003) Usher syndrome type I G (USH1G) is caused by mutations in the gene encoding SANS, a protein that associates with the USH1C protein, harmonin. Hum Mol Genet 12:463-471.

Weisz C, Glowatzki E, Fuchs P (2009) The postsynaptic function of type II cochlear afferents. Nature 461:1126-1129.

Weston MD, Luijendijk MW, Humphrey KD, Moller C, Kimberling WJ (2004) Mutations in the VLGR1 gene implicate G-protein signaling in the pathogenesis of Usher syndrome type II. Am J Hum Genet 74:357-366.

Wever EG, Lawrence M (1948) The functions of the round window. Ann Otol Rhinol Laryngol 57:579-589.

Wise AK, Hume CR, Flynn BO, Jeelall YS, Suhr CL, Sgro BE, O'Leary SJ, Shepherd RK, Richardson RT (2010) Effects of localized neurotrophin gene expression on spiral ganglion neuron resprouting in the deafened cochlea. Mol Ther 18:1111-1122.

Wu WJ, Sha SH, McLaren JD, Kawamoto K, Raphael Y, Schacht J (2001) Aminoglycoside ototoxicity in adult CBA, C57BL and BALB mice and the Sprague-Dawley rat. Hear Res 158:165-178.

Yamasoba T, Yagi M, Roessler BJ, Miller JM, Raphael Y (1999) Inner ear transgene expression after adenoviral vector inoculation in the endolymphatic sac. Hum Gene Ther 10:769-774.

Yan D, Liu XZ (2010) Genetics and pathological mechanisms of Usher syndrome. J Hum Genet 55:327-335.

Yokoi K, Kachi S, Zhang HS, Gregory PD, Spratt SK, Samulski RJ, Campochiaro PA (2007) Ocular gene transfer with self-complementary AAV vectors. Invest Ophthalmol Vis Sci 48(7):3324-8.

Zabner J, Winter M, Excoffon KJ, Stoltz D, Ries D, Shasby S, Shasby M (2003) Histamine alters E-cadherin cell adhesion to increase human airway epithelial permeability. J Appl Physiol. Jul;95(1):394-401.

Zallocchi M, Meehan DT, Delimont D, Askew C, Garige S, Gratton MA, Rothermund-Franklin CA, Cosgrove D (2009) Localization and expression of clarin-1, the Clrn1 gene product, in auditory hair cells and photoreceptors. Hear Res 255:109-120.

Zallocchi M, Meehan DT, Delimont D, Rutledge J, Gratton MA, Flannery J, Cosgrove D (2012) Role for a novel Usher protein complex in hair cell synaptic maturation. PLoS One 7(2):e30573.

Zheng YH, Lovsin N, Peterlin BM (2005) Newly identified host factors modulate HIV replication. Immunol Lett 97:225-234.

Zufferey R, Donello JE, Trono D, Hope TJ (1999) Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. J Virol 73(4):2886-92.